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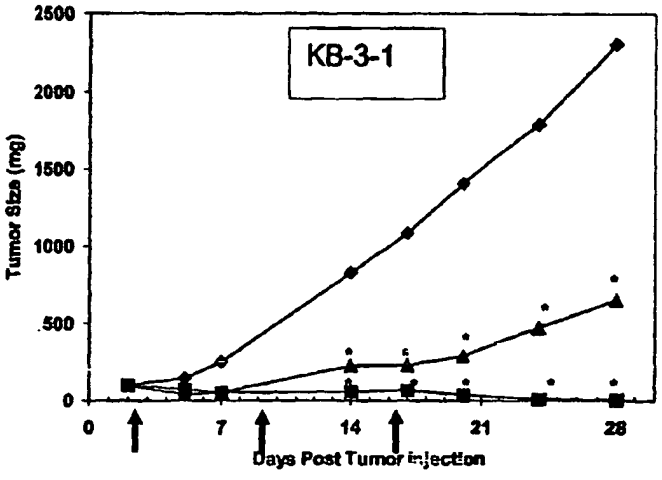
(54) Title: CELLS RESISTANT TO CHEMOTHERAPEUTIC COMPOUNDS AND USES THEREOF



(57) Abstract: The present invention provides cell lines that are resistant to anti-tubulin drugs and, in particular, are resistant to hemiasterlins and/or the hemiasterlin derivative HTI 286. Methods are also provided which use these cell lines to screen for and identify drugs (particularly anti-tubulin drugs) that effectively inhibit the growth and/or replication of cells that are resistant to hemiasterlins and other anti-tubulin drugs such as HTI-286. The invention additionally provides methods that use these cell lines to characterize antitubulin drugs.

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CELLS RESISTANT TO CHEMOTHERAPEUTIC COMPOUNDS AND USES THEREOF

1. CROSS REFERENCE TO RELATED APPLICATIONS

Priority is claimed under 35 U.S.C. 119(e) to copending U.S. provisional patent application Serial No. 60/428,120 filed on November 21, 2002. The contents of this prior application are hereby incorporated by reference and in their entirety.

2. FIELD OF THE INVENTION

The present invention relates to methods and compositions for identifying and characterizing anticancer drugs, particularly anti-tubulin drugs such as hemiasterlins and their derivatives. In particular, the invention provides cell lines that are resistant to hemiasterlin and/or hemiasterlin derivatives and analogues. These include a hemiasterlin analogue referred to here as HTI-286. The invention also relates to methods for using such cells – including methods for identifying new anti-tubulin drugs (including new hemiasterlins, derivatives and analogues) as well as methods for characterizing new and existing anti-tubulin compounds (including hemiasterlins, derivatives and analogues).

3. BACKGROUND OF THE INVENTION

α- and β-tubulin heterodimers polymerize to form microtubules which are vital for mitosis, motility, secretion, and proliferation (Rowinksy and Tolcher, in *Cancer Principles and Practice* (Devita *et al.*, eds.) 6th Ed. 2001, 431-452). Agents that bind tubulin and disrupt the function of microtubules are of great interest as some of these agents are routinely used to treat cancer. Three well-defined classes of drugs that bind tubulin have been previously identified: (1) colchicine, (2) vinca alkaloids and (3) taxanes (Hamel, *Med. Res. Rev.* 1996, 16:207-231). Colchicine and vinca alkaloids bind to distinct sites in tubulin, prevent the formation or extensions of microtubules, and therefore induce the depolymermization of microtubules (Hamel, *Med. Res. Rev.* 1996, 16:207-231; Downing, *Annu. Rev. Cell Dev. Biol.* 2000, 16:89-111). Photoaffinity-labeling and electron microscopy studies have revealed that taxanes bind to a distinct site

within β-tubulin (Downing, *Annu. Rev. Cell Dev. Biol.* 2000, 16:89-111). At stoichiometric amounts in relation to tubulin, taxanes stabilize microtubules and therefore actually enhance polymerization (Jordan, *Curr. Med. Chem. Anti-Canc. Agents* 2002, 2:1-17). However, at low concentrations, which are sufficient to inhibit cell division, all antimicrotubule agents alter microtubule dynamics without causing marked depolymerization or polymerization effects (Jordan, *Curr. Med. Chem. Anti-Canc. Agents* 2002, 2:1-17).

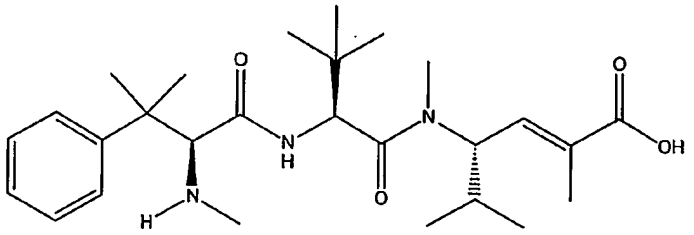
Vinca alkaloids and taxanes such as paclitaxel and docetaxel have been widely used to treat solid tumors. However, resistance to paclitaxel and vinca alkaloids is easily demonstrated in tissue culture systems and occurs frequently either at the onset or during the course of multiple cycles of chemotherapy in patients (Rowinksy and Tolcher, in *Cancer Principles and Practice* (Devita *et al.*, eds.) 6th Ed. 2001, 431-452). In tissue culture systems, vinca alkaloids, paclitaxel, and docetaxel, are excellent substrates for the ABC drug efflux pump, P-glycoprotein. This protein can be overexpressed in tumor cells in response to chemotherapeutic drugs and is believed to mediate resistance to these agents.

Recently a fourth class of anti-tubulin compounds, known as hemiasterlins, has been described. See, for example, Talpir *et al.*, *Tetrahedron Lett.* 1994, 35:4453-4456; Gambel *et al.*, *Bioorg. Med. Chem.* 1999, 7:1611-1615; Coleman *et al.*, *Tetrahedron* 1995, 51:10653-10662; and Anderson *et al.*, *Cancer Chemother. Pharmacol.* 1997, 39:223-226. Hemiasterlins, which are tripeptide compounds isolated from marine sponges, induce microtubule depolymerization, cell cycle arrest and ultimately cell death (Anderson *et al.*, *Cancer Chemother. Pharmacol.* 1997, 39:223-226; Talpir *et al.*, *Tetrahedron Lett.* 1994, 35:4453-4456; Hamel and Covel, *Curr. Med. Chem. Anti-Canc. Agents* 2002, 2:19-53). The use of hemiasterlin compounds in cancer therapy has also been described. See, for example, International Patent Publication Nos. WO 99/32509 and WO 96/33211. See also, U.S. Patent No. 6,153,590. Methods for obtaining hemiasterlin compounds have additionally been described, both by isolating the compounds from marine sponges (U.S. Patent Nos. 5,661,175 and 6,153,590) and by chemical synthesis (Anderson & Coleman, *Tetrahedron Lett.* 1997, 38:317-320).

Synthetic hemiasterlin analogues and derivative compounds have also been described (see, for example, International Patent Publication No. WO 99/32509) and these compounds also have cytotoxic and anti-mitotic activity. In particular, provisional

U.S. patent application Serial Nos. 60/411,883 and 60/493,841 filed on September 20, 2002 and August 8, 2003, respectively, describe various hemiasterlin derivative compounds, including one compound known as HTI-286.

HTI-286, which has the chemical structure set forth in Formula I, below, has a weak interaction with P-glycoprotein and has also been shown to overcome resistance of other anti-tubulin drugs, such as taxanes, both *in vitro* and in xenograft tumor models (Loganzo *et al.*, *Cancer Res.* 2003, 63:1838-1845). Clinical trials of HTI-286 in cancer patients are in progress (Ratain *et al.*, *Am. Soc. Clin. Oncol.* 2003, abstract 516).



(Formula I)

While hemiasterlin and its analogues/derivatives represent a promising class of new anti-tubulin drugs, it is widely accepted that resistance will develop to nearly all chemotherapeutic agents (Cabral, *Drug Resistance Updates* 2000, 3:1-6). Indeed (and as noted above) resistance to paclitaxel and vinca alkoids occurs frequently during chemotherapy in patients (Rowinsky & Torcher, *Cancer Principles and Practice* (Devita *et al.*, eds) 6th Ed. 2001, 431-452). Cultured tumor cell lines have been previously selected for resistance to a variety of different anti-tubulin compounds, including: paclitaxel, epothilones, vinblastine, and colchicine to name a few (Yusuf *et al.*, *Curr. Cancer Drug Targets* 2003, 3:1-19; He *et al.*, *Mol. Cancer Thera.* 2001, 1:3-10; Cabral *et al.*, *Drug Resistance Updates* 2000, 3:1-4). However, cells that are resistant to hemiasterlin drugs have not heretofore been described, and it is therefore not known what mechanisms will give rise to such resistance *in vitro* or *in vivo*.

There is an ongoing need, however, to identify effective therapeutic regimens for individual cancers and, in particular, to identify what tumors are likely to be resistant to particular drugs. Accordingly, there is a continuing need to identify various mechanisms of resistance to chemotherapeutic agents, including resistance to anti-tubulin agents such as to hemiasterlins (including hemiasterlin derivatives or analogues). There is also a need for methods and compositions to characterize different chemotherapeutic agents according to their susceptibility to various types and mechanisms of drug resistance.

* * * * *

The citation and/or discussion of a reference in this section and throughout the specification is provided merely to clarify the description of the present invention and is not an admission that any such reference is "prior art" to the invention described herein.

4. SUMMARY OF THE INVENTION

The present invention provides at least a partial solution to the above problems in the prior art by providing isolated cells and cell lines that are resistant to certain anti-tubulin and other anti-cancer drugs. In particular, the application provides isolated cells that are resistant to one or more hemiasterlin compounds. These hemiasterlin compounds include and/or can be derivatives and analogues of naturally occurring and other hemiasterlins. Hence, in one embodiment the invention provides isolated cells that are resistant to the compound referred to here at HTI-286. In another embodiment, the invention provides isolated cells that are resistant to hemiasterlin A. It will be appreciated that these two embodiments also include isolated cells that are resistant to both these compounds – *i.e.*, isolated cells that are resistant to both HTI-286 and hemiasterlin A.

It is to be understood that, when used to describe and claim cells of the present invention, the term "isolated" indicates that a cell has been removed from its native environment; *i.e.*, from the tissue or cell culture from which it is derived. In particular, cells of the present invention should be sufficiently removed from the tissue and cells from which they are derived such that they can be cultured and manipulated (*e.g.*, by transfection with exogenous nucleic acids and/or treatment with various drugs and other agents) separately from other cells. As such, the term "isolated cell" refers to a cell that is separate from other cells and tissues, as well as referring to a collection of cells (*i.e.*, a "cell line") that share one or more common properties (for example, properties of drug resistance, or the expression of certain nucleic acids and/or proteins) and are grown or cultured separately from other cells not having those properties. Typically, the cells in a cell line or other such collection of cells will be derived from or descendants of a common isolated cell with the properties of cells in the cell line. In addition, cells and cell lines of the present invention are typically progenitors of other cells that have been selected according to one or more particular properties of interest – such as properties of drug resistance and/or nucleic acid or protein expression.

In preferred embodiments, hemiasterlin resistant cells of the invention are isolated from cells that are not normally resistant to that drug, *e.g.*, by selecting cells that proliferate and grow successfully when cultured in some concentration of a hemiasterlin or other drug compound. These cells, from which resistant cells of the present invention are derived, are referred to here as "parental" or "wild-type" cells. In preferred embodiments, the invention provides isolated cells and cell lines that are at least twice, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 times more resistant to a hemiasterlin than the parental cell or cell line from which they are derived.

Generally, a cell's resistance to a hemiasterlin or other drug can be quantitated, *e.g.*, by determining the drug concentration that inhibits growth or proliferation of the cell by some fraction – usually 50% -- compared to the cell growth or proliferation observed in the absence of the drug. This value is known in the art and referred to here as the cell's IC_{50} value or, more precisely, the IC_{50} of the particular drug in a particular cell or cell line. Accordingly, the present invention provides isolated cells and cell lines that have particular IC_{50} values for a hemiasterlin (which can be a hemiasterlin derivative or analogue, as described above). For example, in certain preferred embodiments, the invention provides isolated cells and cell lines in which the IC_{50} value of a hemiasterlin is at least 1.0, 2.0, 5.0, 6, 7, 8, 9, 10, 15, 20, 25 or even 50 nM.

The hemiasterlin resistant cells of the present invention can be resistant to one or more other drugs as well, including but not limited to: microtubule depolymerizing agents (such as HTI-286, hemiasterlin, hemiasterlin analogs, dolastatin-10, dolastatin-15, maytansine, rhizoxin, vinblastine, vinorelbine, phomopsin A, and colchicine), microtubule polymerizing agents (such as paclitaxel, docetaxel, MAC-321, epothilone B, and eleutherobin), and DNA active drugs (such as topotecan, mitoxantrone, camptothecin, adriamycin, and bisantrene). Hence, the invention also provides isolated cells and cell lines that are resistant to both a hemiasterlin (which can be a hemiasterlin analogue or derivative, as described above) and one or more of the other drug compounds mentioned above.

In still other embodiments, the invention provides isolated cells and cell lines that are defined by one or more other unique properties – either in addition to or besides resistance to a hemiasterlin compound. For example, in certain embodiments the invention provides isolated cells and cell lines that, when cultured in the presence of a hemiasterlin (which can be a hemiasterlin derivative or analogue, such as any of those

described *supra*), has an intracellular accumulation of that compound that is lower than the intracellular accumulation in a parent cell or cell line. In preferred embodiments of such cells, the intracellular accumulation of hemiasterlin is at least about 50% lower than in the parental cells. Still other embodiments are contemplated in which the intracellular accumulation of the hemiasterlin compound is at least 60%, 70%, 75%, 80%, 85%, 90%, 95% or 99% lower than in the parental cell line. Preferably, this phenotype is reversible in cells of the invention, *e.g.*, by culturing with a drug or compound (*e.g.*, sodium izide) that inhibits ATP formation in the cells.

In other preferred embodiments, hemiasterlin resistant cells of the present invention can be further characterized by their expression (or lack thereof) of certain, common drug efflux pumps that are normally associated with drug resistance. For exmple, preferred hemiasterlin and other resistant cells of the present invention do not express an ATP-binding cassette (ABC) drug pump, including but not limited to the ABC drug pumps MDR1, MRX, MRP1, and/or MRP3.

Cells and cell lines of the present invention can also be characterized by having and/or expressing one or more mutant nucleic acids that are different from a corresponding sequence in the parent cell or cell line, *e.g.*, by one or more nucleotide insertions, substitutions or deletions. Such mutant nucleic acids may or may not cause drug resistance in those cells (either in whole or in part). Such mutant nucleic acid sequences will typically (but not necessarily) cause a cell to express or produce a mutant (*i.e.*, variant) polypeptide with an amino acid sequence differing from a corresponding amino acid sequence in the parental cell line, *e.g.*, by one or more amino acid substitutions, insertions or deletions. Such cells are therefore also provided in certain embodiments of the invention. For example, in one particularly preferred embodiment, a cell of the invention produces a polypeptide having the amino acid sequence set forth in **Figure 5B** but with one or more amino acid substitutions, deletions or insertions. In a particularly preferred embodiment, a cell of the invention produces a mutant polypeptide having the amino acid sequence set forth in **Figure 5B** but with the amino acid substitution Ala12 → Ser. Similarly, the invention also provides cells that contain (*e.g.*, in their chromosomal DNA) and/or express a variant nucleic acid that encodes a variant of the amino acid sequence set forth in **Figure 5B**, including a variant that encodes the amino acid substitution Ala12 → Ser.

Cells and cell lines of the present invention are particularly useful, *e.g.*, in methods that screen for and/or characterize new drugs including, in particular, anti-tubulin and other anti-cancer drugs. Such methods are therefore also provided in the present invention. For example, in one embodiment the invention provides a method. For example, in certain embodiments, the invention provides methods for characterizing anti-tubulin compounds and other drugs. Generally, such methods involve determining the ability of a test compound to inhibit growth or proliferation of a resistant cell line, and comparing that property to the compound's ability to inhibit growth or proliferation of a non-resistant cell, such as the parental cell line. For example, in preferred embodiments the IC_{50} values for the test compound in a resistant cell line and its parental cell line are determined (*e.g.*, in a cell proliferation assay) and compared.

In other embodiments, the invention provides methods for identifying new compounds that inhibit the growth and/or proliferation of cells that are otherwise resistant to certain drugs. In particular, the invention provides methods for identifying compounds that inhibit the growth and/or proliferation of hemiasterlin resistant cells. Compounds identified in such assays can themselves be useful, *e.g.*, in new cancer therapies and, in particular, for treating drug-resistant cancers. In preferred embodiments, these methods simply involve determining the ability of a test compound to inhibit growth or proliferation of a hemiasterlin resistant cell – for example, by determining the IC_{50} value of the test compound in such a cell (typically by a cell proliferation assay). In preferred embodiments, the test compound's ability is compared to the ability of the hemiasterlin compound (*i.e.*, the compound to which the cell was selected to be resistant) to inhibit growth or proliferation of the resistant cell.

These and other embodiments of the present invention are described in detail in the Sections below.

5. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 demonstrates that KB-HTI-resistant cells retain sensitivity to paclitaxel *in vivo* but are resistant to HTI-286. KB-3-1 **(Figure 1A)** or KB-2.5-HTI **(Figure 1B)** tumor cells were implanted subcutaneously into athymic mice. Animals bearing established tumors were treated intravenously with saline (♦), 1.25 mg/kg HTI-286 (▲), or 60 mg/kg paclitaxel (■). Vehicle and drugs were administered weekly for 3 cycles as

indicated by arrows. Data are the mean of tumor size (milligrams) at each time point. *, P<0.01.

Figures 2A-2C show the analysis of MDR1 (Figure 2A), MXR (Figure 2B), and MRP3 (Figure 2C) drug transport pumps in the KB-3-1 parental cell line and HTI-286-resistant cell lines. Protein levels were determined by SDS-PAGE/immunoblot analyses. RNA levels were evaluated by quantitative RT-PCR as described below and are the mean ± SD of 2 – 4 separate experiments. Positive control cell lines are included for MDR1 (see the KB-8.5 cell line), MXR (see the S1-M1-3.2 cell line), and MRP3 (see the 2008/MRP-3 and A549 cell lines). S1 and 2008 cell lines are negative controls for MXR and MRP3 expression, respectively.

Figure 3 shows data for the drug accumulation of HTI-286 and paxlitaxel in KB-3-1 and HTI-286-resistant cells lines. The indicated cell lines were incubated for 2 hours with [^{3}H]-HTI-286 (■) or [^{14}C]-paclitaxel (□) and levels of radioactive drug remaining in cells were quantified by liquid scintillation counting. Data from four independent experiments are represented as mean percent ± standard deviation of drug remaining in resistant cells as a percentage of the drug in the parental cell line (KB-3-1). The significance of each drug's effect was determined by comparing resistant to parental cells using a two-sided Student's t-test. A significant difference is indicated with a "*" above the bar graph and denotes that the probability value (P) is less than 0.01.

Figure 4 provides data for the drug accumulation of HTI-286 in KB-3-1 and HTI-286-resistant cell lines in the presence of sodium azide. The indicated cell lines were incubated for 2 hours with [^{3}H]-HTI-286 in the absence (■) or presence (□) of 2.5 mM sodium azide and levels of radioactive drug remaining in cells were quantified by liquid scintillation counting. KB-V1 cells, which express high levels of MDR1, are used as a positive control for low drug accumulation that is partially reversible with sodium azide. Data from a representative experiment is indicated as percentage ± standard deviation of drug remaining in resistant cells compared to parental cells (KB-3-1). The significance of each drug's effect was determined by comparing resistant to parental cells using a two-sided Student's t-test. A significant difference is indicated with a "*" above the bar graph and denotes that the probability value (P) is less than 0.01.

Figures 5A-5B list a preferred cDNA sequence (**Figure 5A**) and the amino acid sequence it encodes (**Figure 5B**) for human α-tubulin (GenBank database Accession No. BC017004).

Figure 6A-6B depict a model of the α- and β-subunits in tubulin. **Figure 6B** depicts a close-up view of the same model, showing the location of the Ala12→Ser mutation of α-tubulin in HTI-resistant cells and its proximity to a non-exchanging GTP (N) site at the interface of the α- and β-subunits in tubulin.

Figure 7 shows a protein sequence alignment for amino acid residues 1-451 of human (*Homo sapien,* designated htub1 and htub2), primate (*Macaca fascicularis* and *Macaca mulatta,* designated ptub1 and ptub2 respectively), mouse (*Mus musculus,* designated motub1), hamster (*Cricetulus griseus,* designated hamtub1), rat (*Rattus norvegicus,* designated rattub1), chicken (*Gallus gallus,* designated chicktub1), and frog (*Xenopus laevis,* designated frogtub1) α-tubulin (Stachi *et al.*, *Biochem. Biophys. Res. Commun.* 2000, 270:1111-1118). The EMBL Accession Numbers for htub1, htub2, ptub1, ptub2, motub1, hamtub1, rattub1, chicktub1, and frogtub1 are AJ245922, K00558, X04757, AF141923, AJ245923, M12329, V01227, M16030, and X07046, respectively.

6. DETAILED DESCRIPTION

6.1. Cells resistant to hemiasterlins and their analogs

The present invention provides isolated cells that are generally referred to here as "resistant cells" or "resistant cell lines" and which survive and/or proliferate in concentrations of drugs, in particular anti-cancer drugs, that inhibit the survival and/or proliferation of an appropriate control cell. In particular, cells lines of this invention are resistant to a class of anti-cancer drugs referred to here as "anti-tubulin" drugs or, alternatively as "tubulin inhibitors". Without being limited to any particular theory or mechanism of action, tubulin inhibitors are believed to exert their therapeutic effects by specifically binding tubulin (which can be either α-tubulin or β-tubulin) and disrupting the polymerization of these subunits in microtubules in cells.

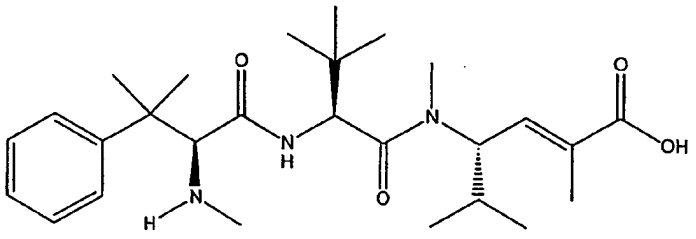
It is to be understood that, when used to describe and claim cells of the present invention, the term "isolated" indicates that a cell has been removed from its native

environment; *i.e.*, from the tissue or cell culture from which it is derived. In particular, cells of the present invention should be sufficiently removed from the tissue and cells from which they are derived such that they can be cultured and manipulated (*e.g.*, by transfection with exogenous nucleic acids and/or treatment with various drugs and other agents) separately from other cells. As such, the term "isolated cell" refers to a cell that is separate from other cells and tissues, as well as referring a collection of cells (*i.e.*, a "cell line") that share one or more common properties (for example, properties of drug resistance, or the expression of certain nucleic acids and/or proteins) and are grown or cultured separately from other cells not having those properties. Typically, the cells in a cell line or other such collection of cells will be derived from or descendants of a common isolated cell with the properties of cells in the cell line. In addition, cells and cell lines of the present invention are typically progenitors of other cells that have been selected according to one or more particular properties of interest – such as properties of drug resistance and/or nucleic acid or protein expression.

Preferred cell lines are resistant to a particular class of anti-tubulin drugs generally referred to as "hemiasterlins." Hemiasterlins are natural products derived from marine sponges that induce microtubule depolymerization, cell cycle arrest and ultimately cell death (Anderson *et al.*, *Cancer Chemother. Pharmacol.* 1997, 39:223-226; Telpin *et al.*, *Tetrahedron Letters* 1994, 35:4453-4456). The use of hemiasterlin compounds in cancer therapy has also been described. See, for example, International Patent Publication Nos. WO 99/32509 and WO 96/33211. See also, U.S. Patent No. 6,153,590. Methods for obtaining hemiasterlin compounds have additionally been described, both by isolating the compound from marine sponges (U.S. Patent Nos. 5,661,175 and 6,153,590) and by chemical synthesis (Anderson & Coleman, *Tetrahedron Letters* 1997, 38:317-320).

Synthetic hemiasterlin analogues and derivative compounds have also been described (see, for example, International Patent Publication No. WO 99/32509) and these compounds also have cytotoxic and anti-mitotic activity. Accordingly, such hemiasterlin derivative and analogue compounds are also considered hemiasterlins, at least for the purposes of describing and claiming this invention. It is to be understood that, for the purposes of describing this invention, the terms "hemiasterlin derivative" and "hemiasterlin analogue" are used interchangeably. Both these terms (in all their variants) therefore generally refer to compounds that are derived from and/or are chemical analogues of a natural hemiasterlin compound.

Particularly preferred classes of hemiasterlin derivatives have been described in provisional U.S. patent application Serial Nos. 60/411,883 and 60/493,841 filed September 20, 2002 and August 8, 2003, respectively. These include a particular hemiasterlin derivative known as HTI-286, which has the chemical structure set forth in Formula I, below.



Formula I

HTI-286 has a weak interaction with P-glycoprotein and has been shown to overcome resistance of other anti-tubulin drugs (*e.g.*, taxanes) both *in vitro* and in xenograft tumor models (Loganzo *et al.*, *Cancer Res.* 2003, 63:1838-1845). The compound is therefore particularly useful as an anti-cancer drug, and clinical trials of HTI-286 in cancer patients are in progress (Ratain *et al.*, *Proc. Am. Soc. Clin. Oncol.* 2003, abstract 516). Hence, cell lines of the present invention that are resistant to HTI-286 are particularly preferred.

Preferred embodiments of the invention include resistant cells that originate from a cell line that is susceptible to (*i.e.* does not survive and/or proliferate in the presence of) one or more anti-tubulin drugs (*e.g.* HTI-286). Such susceptible cell lines, when used to produce one or more drug resistant cell lines of the invention, are referred to here as "parental cells" or "parental cell lines." Resistant cell lines may be created by exposing the parental cell line to increasing concentrations of an anti-tubulin drug. Initially, the parental cell line is exposed to a concentration of drug over time until the cell line is able to survive and/or proliferate at that particular concentration. The cell line is then exposed to a higher concentration of drug (*e.g.* 1.2-1.5 times the pre-existing concentration) until the cells are again able to survive and/or proliferate (*i.e.* adapt). Increasing the drug concentration followed by adaptation to the drug concentration is typically continued until cells can no longer adapt to a concentration despite being given a reasonable period to survive and/or proliferate. Cell lines developed over the course of increasing the concentration of drug may be characterized and used as described below.

Generally speaking, cells of the present invention can be obtained by incubating cells from a parental cell line in the presence of increasing concentrations of a drug or other compound to which resistance is desired – for example in the presence of increasing concentrations of a hemiasterlin such as HTI-286. The starting drug concentration should be a concentration that significantly inhibits, without completely stopping, cell growth and/or proliferation. For example, in a preferred embodiment the starting drug concentration can be equal to or about equal to the drug's IC_{50} value in the parental cell line. That is to say, the drug concentration can be equal to or about equal to the concentration of drug that inhibits cell growth or proliferation by 50%. Those skilled in the art will appreciate that the exact starting drug concentration used is not critical. Hence, the starting drug concentration can also be a concentration that is sufficient to reduce cell growth or proliferation by only 10% (*i.e.*, the IC_{10} value) or by as much as 90% (*i.e.*, the IC_{90} value). Hence, starting drug concentrations that are equal to or about equal to the drug's IC_{10}, IC_{20}, IC_{30}, IC_{40}, IC_{50}, IC_{60}, IC_{70}, IC_{80} or IC_{90} value in the parental cell line can all be used. Examples of some particular starting drug concentrations that can be used are: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 nM. Again, however, it is recognized that the absolute starting concentration is not critical. Hence the values given here are merely approximate, as are other specific values and measurements set forth in this specification. As such, the values can vary in different embodiments, *e.g.*, by as much as 5, 10, or even 20%.

Generally speaking, cells are cultured in a particular concentration of drug at least until the cells are observed to have adapted to the drug and, more specifically, the viability of cells in the presence of the drug has substantially increased (*e.g.*, there is little evidence of cell death in the presence of the drug).

As an example and not by way of limitation, it is well understood in the art that, to maintain cell viability in a cell culture, the cell culture will be passaged from one container to another. Typically, only a fraction and not all of the viable cells in the culture are transferred. However, the fraction of cells transferred or "passaged" in this process should at least be high enough that a viable cell culture can be maintained from the passaged cells. Hence in a typical cell culture (*e.g.*, where there is little or no drug present), the fraction of cells passaged will be less than about 10%, and is more preferably only about 5% or fewer of the viable cells in the culture. However, where a concentration of a drug or other compound inhibiting cell growth is introduced to the

culture, the total number of viable cells is expected to decrease substantially (*e.g.*, by 50% or more) at least until some level of drug resistance occurs. Accordingly, a higher fraction of these cells must be passaged to maintain viable cell cultures (*e.g.*, greater than 10% and more preferably about 20%).

Hence, in a preferred embodiment cell viability can be estimated from the percentage of cells passaged to maintain viable cultures. Once the fraction of passaged cells incubated with a drug is equal to or approximately equal to the fraction passaged to maintain an identical cell culture without drug, it can be said that the cells have adapted to the drug at least enough for culturing in a higher drug concentration.

Once the cultured cells are observed to have at least partly adapted to the initial drug concentration, the drug concentration is increased by some amount and the cells are further cultured in this new, higher drug concentration. Again, the higher drug concentration should be sufficiently high to significantly inhibit cell growth and/or proliferation without completely stopping or abolishing the same. Typically, the drug concentration is increased by a factor of at least 1.1, but higher factors may be preferred. For example, in various embodiments the drug concentration can be increased by a factor of about 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2, 3, 4, 5, 7, 9, or 10.

Generally, any cell line that is susceptible to an anti-tubulin drug (*e.g.*, a hemiasterlin such as HTI-286) can be used as a parental cell line to obtain resistant cells of the invention. However, preferred parental cell lines are eukaryotic cell lines such as vertebrate and mammalian cells. Human cell lines are particularly preferred for parental cell lines of the invention. Generally, any vertebrate or mammalian cell line used for parental cells of the invention will be a cancer cell line (*i.e.*, a cell line produced from cancer cells that are isolated from an individual organism). For instance, one preferred cell line that can be used for parental cells in this invention is a KB cell line. The KB cell line is a human cancer cell line that is known and has been previously described (see, for example, Eagle, *Proc. Soc. Exp. Biol. Med.* 1955, 89:362-384). KB cells are available, for example, from the American Type Culture Collection (ATCC) in Manassas, Virginia (Accession No. CCL-17). Resistant cells of the invention can be derived either directly or indirectly from KB or other parental cells. For example, in a particularly preferred embodiment, resistant cells of the invention are produced from a KB 3-1 cell line which is itself derived from KB cells. The KB 3-1 cell line has been previously described. See,

for example, Cornwell *et al.*, *J. Biol. Chem.* 1986, 261:7921-7928. Akiyama *et al.*, *J. Biol. Somatic Cell Mol. Genet.* 1985, 11:117-126.

In addition to KB cells, resistant cells of the invention can also be derived from other cell lines. For example, over 700 human cancer cell lines are available from the ATCC (Manassas, Virginia) that can be used as parental cell lines in the present invention. Other, preferred cell lines for use in the present invention include human ovarian carcinoma cell lines such as A2780 (obtained from the Biological Testing Branch of the NCI (Frederick, MD)) and 1A9 cells (Eva *et al.*, *Nature* 1982, 295:116-119; Giannakakou *et al.*, *J. Biol. Chem.* 1997, 272:17118-17125). Other cell lines that can be used as parental cell lines of this invention include CHO cells (see, for example, Ling and Thompson, *J. Cell Physiol.* 1974, 83:103-116) and A549 lung carcinoma cells (see, for example, Noskova *et al.*, *Neoplasma* 2002; 49:418-25).

In addition, those skilled in the art can readily establish other cell lines, by isolating cells from an individual organism and culturing the cells according to routine culturing methods and techniques. For instance, cancer cell lines can be established by isolating cancer cells from a sample obtained, *e.g.*, in a biopsy, from a human or other mammalian or vertebrate organism.

Such cancer cells may be obtained from biopsies of a cancer that is or is not already resistant to one or more anti-cancer drugs. For example, in one embodiment the parental cells may be from a cancer that is already resistant to a hemiasterlin (*e.g.*, HTI-286) or other compound to which drug resistance is sought. In another embodiment, the parent cell line may be derived from cells of a cancer that is resistant to some other class or type of anti-cancer drug, such as another type of anti-tubulin drug. For example, the parent cells may be cancer cells that are already resistant to a variety of other classes of anti-tubulin drugs such as colchicine, vinca alkaloids (*e.g.* vinblastine and vinorelbine), and taxanes (*e.g.* paclitaxel, docetaxel, and MAC-321) (Hamel, *Med. Res. Rev.* 1996, 16:207-231).

Generally, resistant cells of the invention can be characterized by the level or degree of their resistance to a particular anti-tubulin drug such as HTI-286. Preferably, the level or degree of a cell line's drug resistance is measured by the drug concentration that inhibits cell growth by about 50% (*i.e.*, the IC_{50} value). It is understood, however, that resistant cell lines can also be defined by or may additionally comprise other properties such as those described in the secions, *infra*. Preferred resistant cell lines of

the invention have IC_{50} values that are at least about 1.2 to 1.5 times greater than the IC_{50} value of the parental cell line from which they are derived. More preferably, resistant cells of the invention are at least twice, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, or 15 times the IC_{50} value of the parental cell line. However, resistant cells having IC_{50} values that are more than 15 times that of the parental cell line are also contemplated, including resistant cells with IC_{50} values that are at least 20, 25, 30, 40, 50, 100, or 1000 times that of the parental cell line. The IC_{50} value of a cell line to a drug or other substance can be readily determined, *e.g.*, in a proliferation assay using techniques such as those described, *infra*.

For instance, the Examples, *infra*, demonstrate one non-limiting embodiment in which cell lines resistant to HTI-286 are developed by continuously exposing KB-3-1 cells to increasing concentrations of that compound. A drug concentration of about 0.7 nM was initially used, which is approximately equal to the concentration that inhibits growth of KB-3-1 cells by 50% (*i.e.* the IC_{50} value). The HTI-286 concentration was successively increased when cells showed at least 50% viability. Viability was determined by counting the number of cells attached to the flask (*i.e.* viable cells) and the number of cells floating in the media in the flask (*i.e.* dead cells). At each higher concentration of HTI-286, cells were passaged as described above. The subsequent HTI-286 concentrations used were approximately 1.2- to 1.5-fold higher in concentration than the pre-existing concentration. Cells that grew well (*i.e.*, with little if any evidence of cell death) in up to 2.5 nM HTI-286 were isolated about five months after initial exposure of the parental KB-3-1 cells line to HTI-286. These cells are designated here as KB-2.5-HTI and are described in more detail below. About ten months after the initial exposure of KB-3-1 cells to HTI-286, other cells were isolated that grew in up to 4.0 nM HTI-286. These cells, designated here as KB-4.0-HTI (and alternatively referred to as KB-4-HTI cells), are also described in more detail below.

As demonstrated above, different resistant cell lines (for example, the KB-2.5-HTI and KB-4.0-HTI cell lines described in the Examples) can be derived sequentially from the same parental cell line after being exposed to varying concentrations of drug. However, resistant cell lines can also be derived from the same or different parental cell line(s) after being exposed to the same or different concentration of drug under the same or different conditions. Additionally, resistant cell lines can be removed from the selective pressure of the drug, and stabilized in an environment where the drug is either not present or is present at a lower concentration. Such cells are also considered part of

the present invention, and may be resistant to either the same or other anti-cancer agents that are used to select the resistant cell line. For instance, the Examples, *infra*, describe another particular embodiment in which drug resistant KB-4.0-HTI cells are removed from media containing HTI-286 and cultured in media that does not contain HTI-286.

5

6.2. Characteristics of cells resistant to hemiasterlins and their analogs

The resistant cells described in Section 6.1 may have, but are not limited to the following characteristics described here.

10 6.2.1 Resistance to one or more tubulin-binding compounds

Resistant cell lines may show resistance to one or more drugs, such as one or more anti-tubulin binding drugs. These drugs include, but are not limited to microtubule depolymerizing agents (such as HTI-286, hemiasterlin, hemiasterlin analogs, dolastatin-10, dolastatin-15, maytansine, rhizoxin, vinbalstine, vinorelbine, phomopsin A, and 15 colchicine), microtubule polymerizing agents (such as paclitaxel, docetaxel, MAC-321, epothilone B, and eleutherobin), and DNA active drugs (such as topotecan, mitoxantrone, camptothecin, adriamycin, and bisantrene).

To test cell lines for their resistance to various compounds, cells lines are typically assayed for their ability to proliferate in the presence or absence of various compounds. 20 Such an assay is generally referred to here as a "cell proliferation assay" or "proliferation assay". A compilation of drugs and their ability to inhibit cell proliferation in a particular cell line or tumor is referred to here as a "resistance profile" for that cell line or tumor, respectively. A proliferation assay initiates by exposing cells in culture to various compounds at varying concentrations. Length of time for exposure to drug may be, for 25 example, between 12 hours and 1 week. 72 hours is a preferred incubation time.

After incubation, cell survival can be assayed according to any technique known to those skilled in the art, such as by the sulforhodamine B (SRB) assay (see Rabindran *et al., Cancer Res.* 1998, 58:5850-5858). Typically, cell survival is quantified by determining the IC_{50} value (*i.e.* the concentration of drug needed to inhibit cell growth by 30 50%). In the proliferation assay, a cell line is resistant to a compound if the cell line shows an increase in proliferation compared to the parental cell line at a particular compound concentration (*e.g.* at the IC_{50} value of the compound in the parental cell line).

Cell line resistance to various drugs can also be assayed *in vivo* by what is referred to here as "*in vivo* efficacy studies." For example, drug resistance can be evaluated in

animal models, where a drug resistant cancer cell is implanted into an animal, such as a mouse. In one preferred embodiment, described in the Examples, *infra*, drug resistant cells of the invention can be implanted subcutaneously into an animal. The implanted cells can then be allowed to develop into a tumor, preferably of some predetermined size, followed by administering to the animal, a drug for which resistance is to be tested. After administering the drug (and preferably, after a time period sufficient for the drug to affect tumor growth) the tumor size is determined, and can be compared, *e.g.*, to the tumor size before administering the drug or, alternatively, to tumor size in an animal that has not received any of the drug. In preferred embodiments, the size of the tumor from a drug resistant cell line is compared to the size of a corresponding tumor from transplanted cells of the parental cell line (which is not drug resistant or is known to be less resistant to the drug) Generally, a tumor from a resistant cell line is considered resistant to a compound if the relative size of the compound-treated tumor from the cell line (compared to an untreated tumor from the same cell line) is larger when compared to the relative size of a compound-treated tumor from the parental cell line (again, compared to an untreated tumor from the same parental cell line). Resistance profiles from *in vivo* efficacy studies are often, but not necessarily, similar to resistance profiles from proliferations assays. Hence drug resistance profiles that are determined both *in vitro* and *in vivo* can be used, for example, to evaluate therapies for treating drug resistant cancers.

6.2.2 Expression of drug efflux pumps

Resistant cell lines of the invention can also express one or more drug efflux pumps and, moreover, the expression of such efflux pumps can contribute at least in part to drug resistance in such cells. Examples of known drug efflux pumps that can be expressed by resistant cells of the invention include ATP-binding cassette (ABC) drug pumps such as MDR1 (which is also referred to as ABCB1), MRX (which is also referred to as ABCG2/BCRP), MRP1, and MRP3 (which is also referred to as ABCC3) (see, Dumontet, *Expert Opin. Investig. Drugs* 2000, 9:779-788). Still more transporters that can be expressed in drug resistant cells include those described by Gottesman *et al.* (*Nature Reviews: Cancer* 2002, 2:48-58), particularly the transporters set forth in Table 1 of that reference.

Expression of drug efflux pumps by resistant and other cell lines can be assayed, *e.g.*, by detecting nucleic acids (such as mRNA) that encode such drug efflux in cells. Alternatively, the expression of drug efflux pumps can also be detected by measuring

such proteins themselves in cells, such as in an immunoassay. Such assays are routine and widely known by those skilled in the art.

As an example, and not by way of limitation, the detection of drug pump nucleic acids can involve contacting and incubating nucleic acids obtained from a sample with one or more labeled nucleic acid reagents, such as recombinant drug pump DNA molecules, cloned genes or degenerate variants thereof, under conditions favorable for specifically annealing or hybridizing these reagents to their complementary sequences in the sample nucleic acids. Preferably the lengths of these nucleic acid reagents are at least 15 to 30 nucleotides. After incubation, all non-annealed or non-hybridized nucleic acids are removed. The presence of nucleic acids that have hybridized, if any such molecules exist, is then detected and the level of drug pump nucleic acid sequences to which the nucleic acid reagents have annealed may be compared to the annealing pattern or level expected from a control sample (*e.g.*, from a parental cell line) to determine whether drug pump nucleic acid is expressed at an elevated level.

In a preferred embodiment of such a detection scheme, the nucleic acid from the cell type of interest may be immobilized, for example, to a solid support such as a membrane or a plastic surface (for example, on a nylon membrane, a microtiter plate or on polystyrene beads). Following immoblization, labeled (*e.g.* radiolabeled and/or fluorescent) drug pump nucleic acid may be exposed to immoblized nucleic acid. After incubation, non-annealed, labeled drug pump nucleic acid reagents may be easily removed and detection of the remaining, annealed, labeled drug pump nucleic acid reagents may be accomplished using standard techniques that are well-known in the art.

Drug pump gene expression assays of the invention may also be performed using *in situ* hybridization (*i.e.*, hybridization directly upon cells, which may be fixed and/or frozen), thereby eliminating the need of nucleic acid purification. Drug pump nucleic acid reagents may be used as probes or as primers for such *in situ* procedures (see, for example, Nuovo, PCR In Situ Hybridization: Protocols And Application, 1992, Raven Press, New York). Alternatively, if a sufficient quantity of the appropriate cells can be obtained, standard Northern analysis can be performed to determine the level of drug pump gene expression by detecting levels of drug pump mRNA.

Alternative methods for the detection of drug pump nucleic acids may involve their amplification, *e.g.*, by PCR (see, for example, the experimental embodiment taught in U.S. Patent No. 4,683,202) followed by detection of the amplified molecules using

techniques that are well known to those of skilled in the art. The resulting level of amplified drug pump nucleic acid may be compared to those levels that would be expected if the sample being amplified contained only normal levels of drug pump nucleic acid, as normal cells or parental cell line, to determine whether elevated levels of a drug pump nucleic acid are expressed.

In one preferred embodiment of such a detection scheme, a cDNA molecule is synthesized from an RNA molecule of interest (*e.g.*, by reverse transcription). A sequence within the cDNA may then be used as a template for a nucleic acid amplification reaction such as PCR. Nucleic acid reagents used as synthesis intitation reagents (*e.g.*, primers) in the reverse transcription and amplification steps of such an assay are preferably chosen from the sequence of the appropriate drug pump or are fragments thereof. Preferably, the nucleic acid reagents are at least about 9 to 30 nucleotides in length. The amplification may be performed using, *e.g.*, radioactively labeled or fluorescently labeled nucleotides, for detection. Alternatively, enough amplified product may be made such that the product can be visualized by standard ethidium bromide or other staining methods.

As an example, and not by way of limitation, the mRNA expression of one or more drug efflux pumps can be determined by quantitative real-time reverse transcription-PCR (RT-PCR) using a quenched fluorescent probe (see Sampath *et al.*, *Mol. Cancer Thera.* 2003, 2:873-884). This techniques was demonstrated in the Examples.

Nucleic acid microarrays are also useful for determining several drug pump gene expression levels in cells during the same assay. Using nucleic acid microarrays, test and control (for example, the parental cell line) mRNA samples from test and control cell samples are reverse transcribed and labeled to generate cDNA probes. The cDNA probes are then hybridized to an array of nucleic acids immobilized on a solid support. The array is configured such that the sequence and position of each member of the array is known. For example, a selection of drug pump genes may be arrayed on a solid support. Hybridization of a labeled probe with a particular array member indicates that the sample from which the probe was derived expresses that gene. If the hybridization signal of a probe from a test (*e.g.* resistant cell line) sample is greater than hybridization signal of a probe from a control (*e.g.* parental cell line) sample, the gene or genes overexpressed in the resistant cell line are identified.

Protein expression levels for drug pumps may also be determined. Analysis of protein expression can proceed by the preparation of cell lysates or extracts such as, cell membranes from a particular cell line followed by detection using antibodies. Detecting the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is assayed by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by assaying the binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

In another example, antibodies or fragments thereof may be employed for *in situ* detection of a drug pump gene product from cells in culture using, for example, immunofluorescence or immunoelectron microscopy techniques. A labeled antibody or antibody fragment is applied by overlaying the labeled antibody or antibody fragment onto a sample. Through the use of such a procedure, it is possible to detect, not only the presence of a drug pump gene product, but also the gene product's distribution in the examined cells. A wide variety of methods that are well known in the art (for example, staining procedures) can be readily modified by those skilled in the art without undue experimentation to achieve such *in situ* detection.

The labeling of cells by antibodies may also be used to detect the presence of a drug pump gene product, for example, by immunofluorescence techniques employing a fluorescently labeled antibody coupled with flow cytometric or cell sorting techniques.

6.2.3 Reduced accumulation of drugs in resistant cells

Without being limited to any particular theory or mechanism of action, drug efflux pumps are believed to cause drug resistance in cells by transporting intracellular drug molecules across the cell membrane and outside of the cell, so that lethal levels of drug do not readily accumulate inside cells. Hence, resistant cells of the present invention can also be characterized, at least in some embodiments, by a lower accumulation of a drug. It is understood that resistant cells of the invention may exhibit drug resistance both in

addition to or alternatively to the expression of any particular drug efflux pump(s). Resistant cells of the invention may exhibit lower intracellular accumulation for any of a variety of different drugs and/or for a combination of different drugs. Such drugs include, *inter alia*, microtubule depolymerizing agents (such as HTI-286, hemiasterlin, hemiasterlin analogs, dolastatin-10, dolastatin-15, maytansine, rhizoxin, vinbalstin, vinorelbine, phomopsin A, and colchicine), microtubule polymerizing agents (such as paclitaxel, docetaxel, MAC-321, epothilone B, and eleutherobin), and DNA active drugs (such as topotecan, mitoxantrone, camptothecin, adriamycin, and bisantrene).

Accumulation of a drug or other compound in a cell can be readily detected by routine techniques that are known in the art and demonstrated in the examples, *infra*. For example, a compound of interest can be detectably labeled such as with a radiolabel (*e.g.*, with ^{3}H, ^{35}S, ^{32}P, ^{125}I, ^{14}C or another radioisotope) or with a fluorescent label (*e.g.* fluorescein, Cy3, Cy5, rhodamine, or another fluorescent label). A resistant or other cell line can then be exposed to the labeled compound, for example by incubating cells with the labeled compound in the culture media. After exposing cells to the labeled compound for time sufficient to allow accumulation in the cells, levels of accumulated compound can be detected, *e.g.*, by measuring the detectable label in cell extracts. Preferably, non-intracellular molecules of the compound are removed (*e.g.*, by washing the cells with media that does not contain the compound) before harvesting cell extracts (*e.g.*, by lysing the cultured cells).

In other embodiments of the invention, drug accumulation levels may be measured (*e.g.*, as described *supra*) in either the presence or in the absence of a compound, such as sodium azide, that inhibits ATP levels in cells. Preferably, the cells are incubated in non-toxic levels of such a compound such that ATP levels are reduced in the cells without substantially inhibiting cell growth. In such embodiments, drug accumulation levels in cells that are exposed to sodium azide (or another ATP inhibiting compound) are preferably compared to drug accumulation levels in cells that are not exposed to the inhibiting compound. Higher drug accumulation levels in cells that are exposed to an ATP inhibiting compound can indicate that the cells express a drug efflux pump, such as any of the pumps described, *supra*, that removes intracellular drug molecules by hydrolyzing ATP.

6.2.4 Mutation in tubulin

In other embodiments, resistant cells of the invention can express tubulin proteins (which can be either α-tubulin, β-tubulin, or both α- and β-tubulin) containing one or more mutations or other polymorphisms. In these and other embodiments, the term "express" or "expression" indicates that information in a gene or DNA sequence contained in the cells can become manifest, for example, by producing RNA (such as rRNA or mRNA) or a protein by activating the cellular functions involved in transcription and translation of a corresponding gene or DNA sequence. A DNA sequence is expressed by a cell to form an "expression product" such as an RNA (e.g., a mRNA or a rRNA) or a protein. The expression product itself, e.g., the resulting RNA or protein, may also said to be "expressed" by the cell. The term "mutant" or "mutated" is used here to describe such genes and gene products that contain any detectable change in their genetic material (for example, in a cell's DNA), as well as referring to any process, mechanism or result of such a change. This includes gene mutations, in which the structure (e.g., DNA sequence) of a gene is altered, any gene or DNA arising from any mutation process, and any expression product (e.g., RNA, protein or enzyme) expressed by a modified gene or DNA sequence. The term "variant" may also be used to indicate a modified or altered gene, DNA sequence, RNA, enzyme, cell, etc.; i.e., any kind of mutant. For example, the present invention relates to altered or "chimeric" RNA molecules that comprise an rRNA sequence that is altered by inserting a heterologous RNA sequence that is not naturally part of that sequence or is not naturally located at the position of that rRNA sequence. Such chimeric RNA sequences, as well as DNA and genes that encode them, are also referred to herein as "mutant" sequences.

Such mutations and polymorphisms include, for example, amino acid substitutions, deletions and insertions, as well as combination thereof. The mutation or polymorphism can, but need not necessarily, effect binding of an anti-tubulin drug (for example, a hemiasterlin such as HTI-286) to tubulin. The mutation or polymorphism can also, but need not necessarily, cause drug resistance (at least in part or to some extent) in such resistant cells.

It is understood that altered protein molecules are usually expressed in cells having one or more mutated genes that encode the altered protein. Hence, resistant cells of the invention can also contain and/or express nucleic acids that encode tubulin and contain one or more mutations or other polymorphisms. Such mutations and polymorphisms can be in nucleic acids encoding either α-tubulin, β-tubulin, or in nucleic

acids encoding both α- and β-tubulin. It is understood that such variant nucleic acids include not only the chromosomal nucleic acids of genes encoding tubulin, but also nucleic acids produced by such genes (for example, mRNA).

As an example, and not by way of limitation, **Figure 5A** shows the sequence of an exemplary nucleic acid that encodes an α-tubulin amino acid sequence (**Figure 5B**) that may be expressed in a parental or non-drug resistant cell line. In one preferred embodiment of the invention, which is demonstrated in the Examples below, a resistant cell line of the invention may express a variant of this nucleic acid, containing one or more amino acid insertions, deletions or substitutions. As a result, the resistant cell preferably produces a variant of the tubulin polypeptide whose amino acid sequence is set forth in **Figure 5B**. In particular, this variant may also contain one or more amino acid insertions, deletions and mutations. In a particularly preferred embodiment, the tubulin polypeptide comprises an amino acid substitution in which amino acid residue 12 (Ala12) is a serine. Hence, in a preferred embodiment the resistant cell expresses a variant of the tubulin nucleic acid set forth in **Figure 5A** in which the codon encoding Ala12 (*i.e.*, the GCT of residues 34-36 in **Figure 5A**) is replaced with a codon that encodes serine (*e.g.*, TCT).

Cell lines express homologous mutations in tubulin are also contemplated and considered part of the present invention. For example, a cell line of the invention may express an α-tubulin polypeptide comprising the amino acid sequence set forth in **Figure 5B** but having an amino acid substitution at residue 12 of that sequence. Although the amino acid substitution Ala12 → Ser is particularly preferred, any of the 19 other naturally occurring amino acid residues may be substituted for Alanine at that position.

Preferably, the substituted amino acid residue differs from Alanine by one or more properties such as hydrophobicity, hydrophilicity, polarity, hydrogen bonding potential, acidic, basic, aromatic and the like. For example, it is will understood in the art that Alanine is a non-polar, hydrophobic amino acid residue (*i.e.*, the side chain of Alanine is hydrophobic and generally uncharged at physiological pH). Hence, the substitution of a hydrophilic or polar and/or charged (*e.g.*, acidic or basic) amino acid residue for Alanine is generally preferred. Examples of hydrophilic amino acid residues that can be substituted for Alanine include Serine and Lysine. Examples of acidic amino acid residues that can be substituted for Alanine include Aspartic acid and Glutamic acid.

Arginine, Lysine and Histidine are all examples of genetically encoded basic amino acid residues that can be substituted for Alanine in tubulin. Genetically encoded polar amino acid residues that can be substituted for Alanine in tubulin include Asparagine and Glutamine.

Although less preferred, substitutions of other hydrophobic, apolar and/or aromatic amino acid residues for Alanine are also contemplated. Examples of hydrophobic amino acid residues that can be substituted for Alanine include Isoleucine, Leucine and Valine. Other apolar amino acid residues that can be substituted for Alanine include Glycine, Proline, Methionine, Leucine, Valine and Isoleucine. Examples of aromatic amino acid residues that can be substituted for Alanine include the amino acids Phenylalanine, Tyrosine and Tryptophan.

Cell lines of the invention may also express a tubulin polypeptide comprising an amino acid sequence that is homologous to the amino acid sequence set forth in **Figure 5B** and comprising a mutation or variation similar to the ones described *supra* for that sequence. It is well known that tubulin amino acid sequences are highly conserved among different isoforms and other homologs of that polypeptide, including homologues from different species organisms, such as among different species of mammals and/or other vertebrates. For example, **Figure 7** provides a sequence alignment for several different polypeptide sequences for α-tubulin and various homologues thereof. Inspection of these figures show that each of these tubulin amino acid sequences comprises an Alanine (which is underlined in **Figure 7**) at a position aligning with and corresponding to Ala12 in the sequence of **Figure 5B**. Hence, cell lines of the present invention may express any one of these or other homologous tubulin polypeptides, with an amino acid substitution, insertion or deletion of the aligning Alanine amino acid residue – including any of the amino acid substitutions, insertions and deletions described *supra*.

Those skilled in the art can also readily align tubulin and other homologous amino acid sequences using routine algorithms such as FASTA (Pearson & Lipman, *Proc. Natl. Acad. U.S.A.* 1988, 85:2444-2448; Pearson, *Methods Enzymol.* 1990, 183:63-98), BLAST (Altschul *et al.*, *Nucl. Acids Res.* 1997, 25:3389-3402; Altschul, *J. Mol. Evol.* 1993, 36:290-300; Altschul *et al.*, *J. Mol. Biol.* 1990, 215:403-410), CLUSTAL and CLUSTALW (Higgins *et al.*, *Nucl. Acids. Res.* 1994, 22:4673-4680), to name a few. Generally, such algnment algorithms will be used with the standard or default parameters, including standard alignment scoring systems and/or a scoring matrix such as

BLOSUM62. See, Henikoff & Henikoff, *Proc. Natl. Acad. Sci.* 1992, 89:10915-10919. However, in certain circumstances that will be appreciated by those skilled in the art, it may be preferable to us nonstandard parameters and/or scoring matrices. For example, in embodiments where very similar amino acid sequences are being compared (such as sequences of α-tubulin) it may be preferably to use a scoring matrix such as BLOSUM90, that has higher cutoffs.

Using alignment algorithms such as those described above, other homologous sequences can be aligned, *e.g.*, to the tubulin amino acid sequence set forth in **Figure 5B** and/or to any of the sequences set forth in **Figure 7**. Amino acid residues that correspond, *e.g.*, to any of the mutations described *supra* (including any of the mutations to Ala12 in **Figure 5B**) can be identified in such an alignment. Hence, cells expressing variant or homologous tubulin sequences that have a mutation at such an aligning residue or residues are also contemplated.

It is understood that, in addition to expressing tubulin polypeptides that have any of the mutations (*e.g.*, any of the amino acid insertions, deletions, and/or substitutions) described, *supra*, cell lines of the invention may also comprise and express one or more nucleic acids that encode such variant tubulin polypeptide sequences. Such cells are therefore considered part of the present invention. Moreover, such variant tubulin amino acid and nucleic acid sequences are themselves. also considered part of the present invention.

The exemplary mutations described here as well as other mutations in a tubulin nucleic acid or protein can be readily detected by techniques that are routine in the art. Mutations maybe in one or more nucleotides in tubulin nucleic acid and/or in one or more residues in tubulin protein.

In one embodiment, any of a variety of sequencing reactions known in the art can be used to directly sequence at least a portion of a tubulin gene (*e.g.*, DNA or cDNA obtained from reverse transcribed mRNA). Mutations can be detected by comparing the sequence of the sample with the corresponding control sequence. Exemplary sequencing reactions include those based on techniques developed by Maxam and Gilbert (*Proc. Natl Acad Sci USA* 1977, 74:560) or Sanger (Sanger *et al*, *Proc. Nat. Acad. Sci* 1977, 74:5463). It is also contemplated that any of a variety of automated sequencing procedures may be utilized when performing the subject assays (*Biotechniques* 1995, 19:448), including sequencing by mass spectrometry (see, for example, U.S. Patent No.

5,547,835 and international patent application Publication Number WO 94/16101 entitled *DNA Sequencing by Mass Spectrometry* by H. Köster; U.S. Patent No. 5,547,835 and international patent application Publication Number WO 94/21822 entitled "DNA Sequencing by Mass Spectrometry Via Exonuclease Degradation" by H. Köster; U.S Patent No.5,605,798 and International Patent Application No. PCT/US96/03651 entitled *DNA Diagnostics Based on Mass Spectrometry* by H. Köster; Cohen *et al.*, *Adv Chromatogr* 1996, 36:127-162; and Griffin *et al.*, *Appl Biochem Biotechnol* 1993, *38*:147-159). It will be evident to one skilled in the art that, for certain embodiments, the occurrence of only one, two or three of the nucleic acid bases need be determined in the sequencing reaction. For instance, A-track or the like, *e.g.*, where only one nucleotide is detected, can be carried out.

Yet other sequencing methods are disclosed, *e.g.*, in U.S. Patent No. 5,580,732 entitled "Method of DNA sequencing employing a mixed DNA-polymer chain probe" and U.S. Patent No. 5,571,676 entitled "Method for mismatch-directed *in vitro* DNA sequencing".

To increase the availability of a gene, it may be necessary to amplify a portion of a tubulin gene prior to identifying the mutations. Amplification can be performed, *e.g.*, by PCR and/or LCR (see Wu and Wallace, *Genomics* 1989, 4:560), according to methods known in the art. In one embodiment, genomic DNA of a cell is exposed to two PCR primers and amplification for a number of cycles sufficient to produce the required amount of amplified DNA. In preferred embodiments, the primers are located between 150 and 350 base pairs apart. Preferred primers are those that amplify the appropriate portion of a tubulin gene.

Alternative amplification methods include: self sustained sequence replication (Guatelli, J.C. *et al.*, *Proc. Natl. Acad. Sci. USA* 1990, 87:1874-1878), transcriptional amplification system (Kwoh, D.Y. *et al.*, *Proc. Natl. Acad. Sci. USA* 1989, 86:1173-1177), Q-Beta Replicase (Lizardi, P.M. *et al.*, *Bio/Technology* 1988, 6:1197), and self-sustained sequence replication (Guatelli *et al., Proc. Nat. Acad. Sci.* 1989, 87:1874), and nucleic acid based sequence amplification (NABSA), or any other nucleic acid amplification method, followed by the detection of the amplified molecules using techniques well known to those of skill in the art. These detection schemes are especially useful for the detection of nucleic acid molecules if such molecules are present in very low numbers.

In some cases, the presence of a specific mutation of a tubulin gene in DNA from a sample can be determined by restriction enzyme analysis. In a further embodiment, protection from cleavage agents (such as a nuclease, hydroxylamine or osmium tetroxide and with piperidine) can be used to detect mismatched bases in RNA/RNA DNA/DNA, or RNA/DNA heteroduplexes (Myers *et al.*, *Science* 1985, 230:1242). See, for example, Cotton *et al*, *Proc. Natl Acad Sci USA* 1988, 85:4397 and Saleeba *et al.*, *Methods Enzymod.* 1992, 217:286-295.

In another embodiment, a mutation can be identified by denaturing high-performance liquid chromatography (DHPLC) (Oefner and Underhill, *Am. J. Human Gen.* 1995, 57:Suppl. A266). In general, PCR products of the DNA of interest can be analyzed by DHPLC. The resulting chromatograms are examined to identify base pair alterations or deletions based on specific chromatographic profiles (see O'Donovan *et al.*, *Genomics* 1998, 52:44-49).

In other embodiments, alterations in electrophoretic mobility are used to identify the type of tubulin mutations. For example, single strand conformation polymorphism (SSCP) may be used to detect differences in electrophoretic mobility between mutant and wild type nucleic acids (Orita *et al.*, *Proc Natl. Acad. Sci USA* 1989, 86:2766, see also Cotton, *Mutat Res* 1993, 285:125-144; and Hayashi, *Genet Anal Tech Appl* 1992, 9:73-79).

In yet another embodiment, the identity of a mutation in tubulin is obtained by analyzing the movement of a nucleic acid containing a potential mutation in polyacrylamide gels containing a gradient of denaturant. This technique is referred to by those skilled in the art as denaturing gradient gel electrophoresis (DGGE) (Myers *et al.*, *Nature* 1985, 313:495; Rosenbaum and Reissner, *Biophys. Chem.* 1987, 265:1275).

Selective oligonucleotide hybridization, selective amplification, or selective primer extension can also be used to detect mutations. For example, oligonucleotide probes may be prepared in which the known mutant nucleotide is placed centrally (mutation-specific probes) and then hybridized to target DNA under conditions which permit hybridization only if a perfect match is found (Saiki *et al.*, *Nature* 1986, 324:163; Saiki *et al.*, *Proc. Natl Acad. Sci USA* 1989, 86:6230; and Wallace *et al.*, *Nucl. Acids Res.* 1979, 6:3543). Such mutation specific oligonucleotide hybridization techniques may be used for the simultaneous detection of several nucleotide changes in different regions of the tubulin gene. For example, oligonucleotides having nucleotide sequences of specific

mutations are attached to a hybridizing membrane and this membrane is then hybridized with labeled sample nucleic acid. Analysis of the hybridization signal will then reveal the identity of the nucleotides of the sample nucleic acid.

Alternatively, mutation specific amplification technology which depends on selective PCR amplification may be used. Oligonucleotides used as primers for specific amplification may carry the mutation of interest in the center of the molecule (so that amplification depends on differential hybridization) (Gibbs *et al.*, *Nucleic Acids Res.* 1989, 17:2437-2448) or at the extreme 3' end of one primer where, under appropriate conditions, mismatch can prevent, or reduce polymerase extension (Prossner, *Tibtech* 1993, 11:238 and Newton *et al.*, *Nucl. Acids Res.* 1989, 17:2503). This technique is also termed "PROBE" for Probe Oligo Base Extension. In addition it may be desirable to introduce a novel restriction site in the region of the mutation to create cleavage-based detection (Gasparini *et al.*, *Mol. Cell Probes* 1992, 6:1).

In another embodiment, identification of a mutation is carried out using an oligonucleotide ligation assay (OLA), as described, *e.g.*, in U.S. Pat. No. 4,998,617 and in Landegren *et al.*, *Science* 1988, 241:1077-1080. Nickerson, D. A. *et al.* have described a nucleic acid detection assay that combines attributes of PCR and OLA (Nickerson *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 1990, 87:8923-8927). In this method, PCR is used to achieve the exponential amplification of target DNA, which is then detected using OLA.

Several techniques based on this OLA method have been developed and can be used to detect specific mutations of a tubulin gene. For example, U.S. Patent No. 5593826 discloses an OLA using an oligonucleotide having 3'-amino group and a 5'-phosphorylated oligonucleotide to form a conjugate having a phosphoramidate linkage. In another variation of OLA described in Tobe *et al.* (*Nucleic Acids Res* 1996, 24:3728), OLA combined with PCR permits typing of two different mutations in a single microtiter well.

Single base mutations can also be detected. In one embodiment, the single base polymorphism (*i.e.* mutants) can be detected by using a specialized exonuclease-resistant nucleotide, as disclosed, *e.g.*, in Mundy, C. R. (U.S. Pat. No. 4,656,127). According to the method, a primer complementary to the sequence immediately 3' to the mutation site is permitted to hybridize to a target molecule obtained from a particular animal or human. If the mutation site on the target molecule contains a nucleotide that is complementary to the particular exonuclease-resistant nucleotide derivative present, then that derivative will

be incorporated onto the end of the hybridized primer. Such incorporation renders the primer resistant to exonuclease, and thereby permits its detection. Since the identity of the exonuclease-resistant derivative of the sample is known, a finding that the primer has become resistant to exonucleases reveals that the nucleotide present in the mutation site of the target molecule was complementary to that of the nucleotide derivative used in the reaction. This method has the advantage that it does not require the determination of large amounts of extraneous sequence data.

In another embodiment of the invention, a solution-based method is used for determining the identity of the nucleotide of a mutation site. Cohen, D. *et al.* (French Patent 2,650,840; PCT Appln. No. WO91/02087). As in the Mundy method of U.S. Pat. No. 4,656,127, a primer is employed that is complementary to sequences immediately 3' to a mutation site. The method determines the identity of the nucleotide of that site using labeled dideoxynucleotide derivatives, which, if complementary to the nucleotide of the mutation site will become incorporated onto the terminus of the primer.

An alternative method, known as Genetic Bit Analysis ("GBA") is described by Goelet, P. *et al.* (PCT Appln. No. 92/15712). The method of Goelet, P. *et al.* uses mixtures of labeled terminators and a primer that are complementary to the sequence 3' to a mutation site. The labeled terminator that is incorporated is thus determined by, and complementary to, the nucleotide present in the mutation site of the target molecule being evaluated. In contrast to the method of Cohen *et al.* (French Patent 2,650,840; PCT Appln. No. WO91/02087) the method of Goelet, P. *et al.* is preferably a heterogeneous phase assay, in which the primer or the target molecule is immobilized to a solid phase.

Recently, several primer-guided nucleotide incorporation procedures for assaying mutation sites in DNA have been described (Komher *et al.*, *Nucl. Acids. Res.* 1989, 17:7779-7784; Sokolov, *Nucl. Acids Res.* 1990, 18:3671; Syvanen *et al.*, *Genomics* 1990, 8:684-692; Kuppuswamy *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* 1991, 88:1143-1147; Prezant *et al.*, *Hum. Mutat.* 1992, 1:159-164; Ugozzoli *et al.*, *GATA* 1992, 9:107-112; Nyren *et al.*, *Anal. Biochem* 1993, 208:171-175). These methods differ from GBA™ in that they all rely on the incorporation of labeled deoxynucleotides to discriminate between bases at a mutation site.

For determining the identity of mutations located in the coding region of a tubulin gene, yet other methods than those described above can be used. For example, identification of a mutated tubulin protein can be performed by using an antibody

specifically recognizing the mutant protein in, *e.g.*, immunohistochemistry or immunoprecipitation. Antibodies to wild-type tubulin protein or mutated forms of tubulin proteins can be prepared according to methods known in the art. These specific antibodies may be labeled with, for example, a radiolabel and/or a fluorescent marker.

Detecting the desired antibody can be accomplished by techniques known in the art, *e.g.*, radioimmunoassay, ELISA (enzyme-linked immunosorbant assay), "sandwich" immunoassays, immunoradiometric assays, gel diffusion precipitin reactions, immunodiffusion assays, precipitation reactions, agglutination assays (*e.g.*, gel agglutination assays, hemagglutination assays), complement fixation assays, immunofluorescence assays, protein A assays, and immunoelectrophoresis assays, etc. In one embodiment, antibody binding is assayed by detecting a label on the primary antibody. In another embodiment, the primary antibody is detected by assaying the binding of a secondary antibody or reagent to the primary antibody. In a further embodiment, the secondary antibody is labeled. Many means are known in the art for detecting binding in an immunoassay and are within the scope of the present invention.

Immunofluorescence techniques employing a fluorescently labeled antibody coupled with light microscopic, flow cytometric, or fluorimetric detection may also be used to detect mutations. The antibodies (or fragments thereof) useful in the present invention may, additionally, be employed histologically, as in immunofluorescence or immunoelectron microscopy, for *in situ* detection of tubulin mutations in cells. The antibody (or fragment) is preferably applied by overlaying the labeled antibody (or fragment) onto a biological sample. Through the use of such a procedure, it is possible to determine not only the presence of the tubulin polypeptide, but also its distribution in the examined cells. Using the present invention, one of ordinary skill will readily perceive that any of a wide variety of histological methods (such as staining procedures) can be modified in order to achieve such *in situ* detection.

It is understood that, for purposes of describing the present invention, the term mutation refers not only to a gene or other nucleic acid with one or more sequence variations (*e.g.*, nucleotide substitutions, deletions or insertions). The term mutation, as used here, can also refers to a protein or other gene product encoded by such a mutant nucleic acid and which can, preferably, itself contain one or more sequence variations (*e.g.*, one or more amino acid substitutions, deletions or insertions).

6.3. USES OF CELL LINES RESISTANT TO HEMIASTERLINS

6.3.1 Characterizing tubulin-binding compounds

Resistant cell lines and parental cells of the present invention are useful, *inter alia*, in screening assays and methods to characterize compounds and, in particular, to characterize anti-tubulin and other anti-cancer drugs. For example, cell lines of the present invention can be used to characterize mechanisms of interaction for anti-tubulin and other compounds and, in particular, to determine whether such compounds either bind tubulin or inhibit cell growth by the same, similar or different mechanisms than, *e.g.*, a hemiasterlin compound such as HTI-286. Accordingly, the invention provides novel methods using cell lines to characterize drugs and other compounds.

Compounds whose activity can be characterized using cell lines of the invention include, but are not limited to, small molecules (*e.g.*, organic or inorganic molecules which are less than about 2 kDa in molecular weight, are more preferably less than about 1 kDa in molecular weight, and/or are able to cross the blood-brain barrier and affect tubulin or activities associated therewith) as well as macromolecules (*e.g.*, molecules greater than about 2 kDa in molecular weight). Compounds used to inhibit binding may also include peptides and polypeptides. Examples of such compounds (including peptides) include but are not limited to: soluble peptides; fusion peptide members of combinatorial libraries (such as ones described by Lam *et al.*, *Nature* 1991, 354:82-84; and by Houghten *et al.*, *Nature* 1991, 354:84-86); members of libraries derived by combinatorial chemistry, such as molecular libraries of D- and/or L-configuration amino acids; phosphopeptides, such as members of random or partially degenerate, directed phosphopeptide libraries (see, *e.g.*, Songyang *et al.*, *Cell* 1993, 72:767-778); antibodies, including but not limited to polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies; antibody fragments, including but not limited to Fab, $F(ab')_2$, Fab expression library fragments, and epitope-binding fragments thereof.

Preferably, compounds that are characterized in such assays are compounds that have been previously identified as anti-cancer compounds. More preferably, compounds tested in such assays are ones that have been previously identified as anti-tubulin compounds. However, it is not essential for a compound tested in such assays to have been previously either as an anti-cancer or an anti-tubulin compound. Indeed, using the assays of this invention it is possible for one skilled in the art to determine whether a

compound has properties (*e.g.*, the inhibition of cell growth and/or proliferation) that may make it a an anti-tubulin or other anti-cancer compound. Hence, the invention additionally provides assays for characterize compounds as either anti-cancer or anti-tubulin compounds.

Generally, a compound of interest can be characterized in the present invention by determining its ability to inhibit the growth or proliferation of a resistant cell line – that is to say, the growth or proliferation of a cell line of the present invention that is resistant to a particular anti-tubulin or other anti-cancer drug. In preferred embodiment, the resistant cell line is resistant to one or more hemiasterlin compounds, such as HTI-286. For example, the cell lines KB-2.5-HTI and KB-4.0-HTI (described in the examples, *infra*) can be used in an assay of the invention.

The ability of a compound to inhibit the growth or proliferation of a cell line can be readily determined using techniques well known in the art. For example, in one preferred embodiment, the ability of a compound to inhibit cell growth and/or proliferation can be determined in a proliferation assay, as demonstrated in the Examples, *infra*.

Preferably, when characterizing a compound using a resistant cell line, the compound's ability to inhibit growth and/or proliferation of a non-resistant cell line (*e.g.*, a parental cell line) is also determined, and/or this information is obtained by the user. Where a compound effectively inhibits growth or proliferation of the non-resistant or parental cell line but does not inhibit growth or proliferation of the resistant cell line, then that compound is identified as having a mechanism of action that is either the same as or similar to the mechanism of action for that compound against which drug resistance has been selected. As an example and not by way of limitation, where a compound is characterized using a hemiasterlin-resistant cell line (*i.e.*, using a cell line that has been selected for resistance to a hemiasterlin compound such as HTI-286), then a compound which inhibits growth or proliferation of the parental cell line but does not inhibit the growth or proliferation of the hemiasterlin resistant cell line can be said to have a mechanism of action that is the same as or at least similar to the mechanism of action for a hemiasterlin compound such as HTI-286.

It is understood that a compound tested according to these methods can be determined to inhibit the growth and/or proliferation of a resistant cell line, but nevertheless does so at a much lower level than it inhibits growth or proliferation of the

non-resistant cell line (*e.g.*, the parental cell line). For example, the concentration of compound at which cell growth and/or proliferation is reduced by 50% (*i.e.*, the IC_{50} value) may be substantially higher in a resistant cell line of the invention than in a parental cell line. In such embodiment, the compound can still be characterized as having the same or a similar mechanism of action as the compound against which drug resistance was selected.

In preferred embodiments of these methods, a compound may be characterized by comparing its activity (*e.g.*, its effects on cell growth and/or proliferation) in several different cell lines that, preferably, have been selected for resistance against several different drugs. For example, cell lines that are resistant to other anti-tubulin compounds (paclitaxel, epothilones, vinblastine, and colchicine, to name a few) are known and/or readily available to those having ordinary skill in the art (He *et al.*, *Mol. Cancer Thera.* 2000, 1:3-10; Giannakakou *et al.*, *J. Biol. Chem.* 1997, 272:17118-17125; Cabral *et al.*, *Proc. Natl. Acad. Sci. USA* 1981, 78:4399-4391; Cabral *et al.*, Cell 1980, 20:29-36). Hence, a compound of interest can be characterized, *e.g.*, by measuring its effect on cell growth and/or proliferation assay, in one or more of such different resistant cell lines. Preferably, the different cell lines include cells that are resistant to different classes of anti-tubulin compounds. Such cell lines include, *inter alia*, cells that are resistant to one or more microtubule depolymerizing agents (for example, HTI-286, hemiasterlin, hemiasterlin analogs, dolastatin-10, dolastatin-15, maytansine, rhizoxin, vinbalstin, vinorelbine, phomopsin A, and colchicine), cells that are resistant to one or more microtubule polymerizing agents (such as paclitaxel, docetaxel, MAC-321, epothilone B, and eleutherobin), and cells that are resistant to DNA active drugs (such as topotecan, mitoxantrone, camptothecin, adriamycin, and bisantrene).

Generally, it is understood that a compound affecting the growth or proliferation of cells resistant to one type of anti-tubulin or anti-cancer compound may not affect the growth or proliferation of cells that are resistant to another type of anti-tubulin or anti-cancer compound. As an example and not by way of limitation, a compound of interest that affects the growth or proliferation of a hemiasterlin resistant cell line (for example, a cell line selected for resistance to HTI-286) may not affect the growth or proliferation of a cell line that has been selected for resistance to some other anti-cancer compound -- for example, a cell line resistant to paclitaxel or some other tubulin polymerizing agent) – and *vice versa*. Hence, by comparing a compound's effects on the growth and/or

proliferation of a plurality of different cell lines, each of which is preferably selected for resistance to a different anti-tubulin or anti-cancer drug, it is expected that the compound's mechanism(s) of action can be more precisely characterized.

6.3.2 Identifying compounds that inhibit the growth of hemiasterlin resistant cell lines

Cell lines and assays of the present invention can also be used to identify compounds that are effective against cells (particularly cancer cells) that are otherwise drug resistant cells. For example and not by way of limitation, using a cell line selected against a hemiasterlin drug such as HTI-286 (for example, the cell lines KB-2.5-HTI and/or KB-4.0-HTI described *infra*) it is possible to identify compounds, including other hemiasterlin compounds (*e.g.*, hemiasterlin derivatives and analogues) that effectively inhibit the growth and/or proliferation of hemiasterlin resistant cells. Such compounds can themselves be useful, *e.g.*, in novel therapies for treating cancer and, in particular, for treating drug resistant cancers and tumors against which other more traditional therapies may not be effective.

Generally, such assays simply involve determining the ability of a test compound to inhibit cell growth and/or proliferation (*e.g.*, in a proliferation assay as described *supra* and as demonstrated in the Examples) in a resistant cell. In preferred embodiments, the level or degree by which the test compound inhibits growth or proliferation of the resistant cells can be compared to the level or degree by which another compound inhibits the growth and/or proliferation of those cells. In particularly preferred embodiments, the test compound's ability to inhibit growth or proliferation of a resistant cell line is compared to the ability of a compound against which drug resistance has been selected to inhibit growth and/or proliferation of the resistant cell line. For example and not by way of limitation, a test compound may be tested in an assay to determine its ability to inhibit the growth or proliferation of a cell line selected for resistance to a hemiasterlin compound, such as HTI-286. In preferred embodiments, the test compound's ability to inhibit the growth or proliferation of those cells is compared to the ability of the hemiasterlin compound (*e.g.*, HTI-286) to inhibit growth and/or proliferation of those cells. For example, in particularly preferred embodiments the IC_{50} value of both compounds (*i.e.*, the concentration of both compounds that inhibits cell growth and/or proliferation by 50%) is determined or otherwise obtained, and these values can then be compared. A compound that inhibits growth and/or proliferation of the resistant cell line

(as evidence, for example, by having a lower IC_{50} value) is then identified as a compound that is effective against a drug resistant cell. Such a compound can itself be useful, *e.g.*, in novel therapies for treating cancer and, in particular, for treating drug resistant cancers and tumors against which other, more traditional therapies may not be effective.

Any type of compound that can be tested in other screening assays of the invention can also be used in the assays described here. Hence, classes of compounds that can be used in these assays include, but are not limited to, small molecules (*e.g.*, organic or inorganic molecules which are less than about 2 kDa in molecular weight, are more preferably less than about 1 kDa in molecular weight, and/or are able to cross the blood-brain barrier and affect tubulin or activities associated therewith) as well as macromolecules (*e.g.*, molecules greater than about 2 kDa in molecular weight). Compounds used to inhibit binding may also include peptides and polypeptides. Examples of such compounds (including peptides) include but are not limited to: soluble peptides; fusion peptide members of combinatorial libraries (such as ones described by Lam *et al.*, *Nature* 1991, 354:82-84; and by Houghten *et al.*, *Nature* 1991, 354:84-86); members of libraries derived by combinatorial chemistry, such as molecular libraries of D- and/or L-configuration amino acids; phosphopeptides, such as members of random or partially degenerate, directed phosphopeptide libraries (see, *e.g.*, Songyang *et al.*, *Cell* 1993, 72:767-778); antibodies, including but not limited to polyclonal, monoclonal, humanized, anti-idiotypic, chimeric or single chain antibodies; antibody fragments, including but not limited to Fab, $F(ab')_2$, Fab expression library fragments, and epitope-binding fragments thereof.

Preferred compounds that may be used to assay cell survival and/or growth include, but are not limited to microtubule depolymerizing agents (such as HTI-286, hemiasterlin, hemiasterlin analogs, dolastatin-10, dolastatin-15, maytansine, rhizoxin, vinbalstin, vinorelbine, phomopsin A, and colchicine), microtubule polymerizing agents (such as paclitaxel, docetaxel, MAC-321, epothilone B, and eleutherobin), and DNA active drugs (such as topotecan, mitoxantrone, camptothecin, adriamycin, and bisantrene).

In preferred embodiments, compounds that are tested in such assays are themselves derivatives or analogues of the compound against which drug resistance has been selected in the resistant cell line. Alternatively, such compounds may have the same or a similar mechanism of action as the compound against which drug resistance has been selected. As an example and not by way of limitations, in embodiments where a

hemiasterlin resistant cell line is used (for instance, the cell line KB-2.5-HTI, KB-4.0-HTI and/or another cell line selected for resistance to the hemiasterlin derivative HTI-286), a compound tested in the present assay is also preferably a hemiasterlin compound and may be, for example, a novel hemiasterlin derivative or analogue.

7. EXAMPLES

The present invention is also described and demonstrated by way of the following examples. However, the use of these and other examples anywhere in the specification is illustrative only and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to any particular preferred embodiments described here. Indeed, many modifications and variations of the invention may be apparent to those skilled in the art upon reading this specification, and such variations can be made without departing from the invention in spirit or in scope. The invention is therefore to be limited only by the terms of the appended claims along with the full scope of equivalents to which those claims are entitled.

7.1. Materials and Methods

7.1.1 Reagents

HTI-286 was synthesized according to methods that are described elsewhere (Loganzo *et al.*, *Cancer Res.* 2003, 63:1838-1845; Nieman *et al.*, *J. Nat. Prod.* 2003, 66:183-199). Various other chemotherapeutic reagents used in these experiments are also readily available from commercial suppliers. For example, paclitaxel, vincristine, vinblastine, colchicine and doxorubicin are available from Sigma (St. Louis, MO). MAC-321 is available from Taxolog, Inc. (Fairfield, NJ). Docetaxel and vinorelbine are available from MedWorld Pharmacy (Valley Cottage, NY). Dolastatin-15, epothilone B and phomopsin A are available from Calbiochem (San Diego, CA).

Tritium (^{3}H) labeled HTI-286 (74 mCi/mmol) was prepared using standard methods and/or custom-radiolabeled by Moravek Biochemicals, Inc. (Brea, CA) or PerkinElmer Life Sciences (Boston, MA). Carbon-14 (^{14}C) labeled paclitaxel (74 mCi/mmol) was obtained from Moravek Biochemicals, Inc. (Brea, CA). Non-radioactive compounds were solubilized as 1 ot 10 mM stocks in dimethyl sulfoxide (DMSO) for *in vitro* studies. Sodium azide was obtained from Sigma (St. Louis, MO).

7.1.2 Selection of HTI-286 Resistant Cells

A parental KB-3-1 cell line (Cornwell *et al.*, *J. Biol. Chem.* 1986, 261:7921-7928; Akiyama *et al.*, *Somatic Cell Mol. Genet.* 1985, 11:117-126) was maintained as described elsewhere (Loganzo *et al.*, *Cancer Res.* 2003, 63:1838-1845). To produce HTI-resistant cells, the parental KB-3-1 cells were incubated with step wise-increasing concentrations of HTI-286 starting with a concentration of 0.7 nM and subsequent steps using 1.0, 1.2, 1.5, 2.0, 2.5, 3.0, 4.0 and 6.0 nM of that drug (*i.e.*, approximately 1.2- to 1.5-fold increases). Cells were successively passed to the next higher drug concentration when they had apparently adapted to the current concentration, as indicated by more rapid growth. Cells adapted to growth in 2.5 nM of HTI-286 (designated KB-2.5-HTI cells) and in 4.0 nM HTI-286 (designated KB-4.0-HTI cells) were isolated and their drug resistance profile(s) studied. A subset of KB-4.0-HTI cells was also isolated, removed from HTI-286 and maintained in normal (*i.e.*, drug free) growth media. This cell line is designated KB-4.0-HTI(-). Resistance profiles for KB-4.0-HTI(-) cells were determined 3 to 7 months after the cells had been removed from HTI-286 containing media.

A parallel series of KB-3-1 parental cells was also incubated with step-wise increasing concentrations of paclitaxel up to 15 nM. These cells, which are designated KB-15.0-PTX cells, are described elsewhere (Loganzo *et al.*, *Proc. Amer. Assoc. Cancer Res.* 2003, 44:1152 (abstract #5770)).

7.1.3 Cell Proliferation Assays

Cell proliferation studies were performed according to procedures described by Loganzo *et al.* (*Cancer Res.* 2003, 63:1838-1845; also see Rabindran *et al.*, *C. Cancer Res.* 1998, 58:5850-5858). Briefly, cells were plated in 96-well plates in 100 µL media at densities pre-determined to produce 60-90% confluence at the time of analysis. Compounds, which were serially diluted into media as 2x stocks, were added to cells in duplicate. After 72 hours of incubation, cell survival was assayed by the SRB assay as described (Rabindran *et al.*, *C. Cancer Res.* 1998, 58:5850-5858). HTI-286 was not added to the media of HTI-286-resistant cells during the 72 hour treatment. IC_{50} values (*i.e.* the concentration of drug needed to inhibit cell growth by 50%) were used to evaluate drug potency.

7.1.4 *In Vivo* efficacy studies

Athymic *nu/nu* female mice, 5-6 weeks of age were obtained from Charles River Laboratories (Wilmington, MA). The flanks of nude mice were implanted subcutaneouly with 2.5 x 10^6 KB-3-1 or HTI-286-resistant cells. When tumors attained a mass of between 80 and 120 mg (day 0), animals were randomized into treatment groups. After randomization, animals were treated intravenously with 1.25 mg/kg HTI-286 prepared in saline, 60 mg/kg/dose paclitaxel prepared in 6% ethanol/ 6% Cremophor EL/ 88% saline, or a saline vehicle control. Doses were approximately 80% of the maximum tolerated dose (Loganzo *et al., Cancer Res.* 2003, 63:1838-1845). Drugs were administered approximately once a week for 3 weeks. Tumor size was calculated ([*length* x *width*^{2}] /2) and data were analyzed by a two-sided Student's t-test.

7.1.5 Quantification of MDR1 and MXR mRNA

Levels of mRNA for drug transporter proteins in parental and resistant cell lines were determined by quantitative real-time reverse transcription-PCR (RT-PCR) as described elsewhere (Sampath *et al., Mol. Cancer Thera.* 2003, 2:873-884). Oligonucleotide primers and probes for MDR1 and MXR were designed using Primer Express software version 1.0 from PE Applied Biosystems (Foster City, CA) using the published GenBank sequence of human MDR1 (accession number M14758) and human MXR/ABCG2 (accession number XM_032424).

7.1.6 Analysis of MDR1, MXR, and MRP3 protein expression

Levels of MDR1, MXR, and MRP3 protein in cell lines were determined by isolating cell membranes, followed by standard SDS/PAGE, immunoblot, and chemiluminescent analyses as described elsewhere (Loganzo *et al., Cancer Res.* 2003, 63:1838-1845). MDR1, MXR, and MRP3 were detected with PC03 antibodies from Oncogene Science (Uniondale, NY) at a 1:500 dilution, BXP-21 antibodies (Maliepaard *et al., Cancer Res.* 2001, 61:3458-3464) at a 1:50 dilution, and M_3II-9 antibodies from Chemicon (Temecula, CA) (also, see Scheffer *et al., Cancer Res.* 2000, 60:5269-5277) at a 1:50 dilution, respectively. Exposure to protein-specific antibodies was followed by detection with goat-anti-rabbit (in the case of MDR1 and MXR) or goat anti-mouse (in the case of MRP3) IgG- horseradish peroxidase conjugated secondary antibody from

BioRad (Hercules, CA) at a 1:1000 dilution. Chemiluminescence reagents from Amersham (Piscataway, NJ) were used to enhance signal from these blots.

7.1.7 Drug accumulation studies

Cells were plated in triplicate wells of a 24-well dish ($2x10^5$ cells/well) and grown overnight in the absence of any drugs. After a wash with pre-warmed serum-free media, 1 nM [^{3}H]-HTI-286 (about 0.008 uCi) or 500 nM [^{14}C]-paclitaxel (about 0.01 uCi) in serum-free media was added. Concentrations of HTI-286 near the IC_{50} for growth inhibition of parental and HTI-resistant KB cells (0.5 to 6 nM) could be used because of the high specific activity of [^{3}H]-HTI-286. After the addition of drugs, cells were incubated at 37 °C for 2 hours. Drug-containing media was then aspirated and cells were washed 3 times with cold phosphate buffered saline (PBS). Cells were lysed in 2 N NaOH for 1 hour at room temperature. An aliquot of lysed material was removed, neutralized with an equal volume of 2 N HCl, mixed with scintillation cocktail, and counted. The protein content in each sample was quantified using the BioRad (Hercules, CA) DC protein assay. Inhibition of ATP-dependent transporters was accomplished by incubating cells in a non-toxic concentration (2.5 mM) of sodium azide in glucose-free media (Skovsgaard, *Cancer Res.* 1978, 38: 1785-1791).

7.1.8 Sequencing tubulin cDNA

Total RNA was isolated using the RNAgents Total Isolation System from Promega, (Madison, WI) and cDNA was obtained with the SuperScript First-Strand Synthesis System from Invitrogen (Carlsbad, CA). Tubulin transcripts were amplified using Pfx DNA polymerase from Invitrogen and 5'- and 3'-terminal primers specific for class I (hM40) human β-tubulin (GenBank accession number J00314) and human α1-tubulin (GenBank accession number BC017004). PCR products were gel-purified using the QIAquick Gel Extraction kit from Qiagen (Valencia, CA) and sequenced on an ABI 3700 capillary array sequencer from Applied Biosystems, Inc. (Foster City, CA) using primers specific for the respective gene sequences. The following forward and reverse primers were used:

α1-tubulin forward primers:

TGAGTGCATCTCCATCCACG	(Seq ID No: 1)
TCCACCCTGAGCAGCTCATC	(Seq ID No: 2)
AGGTTTCCACAGCTGTAGTTGAG	(Seq ID No: 3)
ACTTCCCTCTGGCCACATATG	(Seq ID No: 4)
TTCAAGGTTGGCATCAACTACC	(Seq ID No: 5)

α1-tubulin reverse primers:

CAATGACTGTGGGTTCCAAG	(Seq ID No: 6)
ACTGACAGGCGTTCCATGAG	(Seq ID No: 7)
GGAAGCAGTGATGGAGGACAC	(Seq ID No: 8)
ACGGTACAACAGGCAGCAAG	(Seq ID No: 9)
CACACAGCTCTCTGTACCTTGG	(Seq ID No: 10)
CTCTCCTTCTTCCTCACCCTC;	(Seq ID No: 11)

class I (hM40) human β-tubulin forward primers:

ACCAGATCGGTGCCAAGTTC	(Seq ID No: 12)
AGGTAACAACTGGGCCAAAG	(Seq ID No: 13)
CCCTCTCCGTCCATCAGTTG	(Seq ID No: 14)
TCTTTATGCCTGGCTTTGCC	(Seq ID No: 15)
CAGCTACTTTGTGGAATGGATC	(Seq ID No: 16)

class I (hM40) human β-tubulin reverse primers:

CATGGTCCCAGGTTCTAGATCC	(Seq ID No: 17)
TCTGCCTCCTTCCGTACCAC	(Seq ID No: 18)
CCTCGTTGTCAATGCAATAGG	(Seq ID No: 19)
CGATACTGCTGGCTTCCACG	(Seq ID No: 20)
CGAGGTGGGATGTCACAGAC	(Seq ID No: 21)
CCTCCTCACCGAAATCCTCC	(Seq ID No: 22)

7.2. Selection of HTI-286 Resistant Cell Lines

KB-3-1 cells were selected for resistance to HTI-286 by continuously exposing growing cells to increasing concentrations of HTI-286. The initial selection concentration of HTI-286 was 0.7 nM, which is approximately equal to the IC_{50} for KB-3-1 cells after 3 day growth exposure. The HTI-286 concentration was successively increased when cells appeared to adapt and rapidly grow at a drug concentration. The subsequent HTI-286 concentrations used were 1.0, 1.2, 1.5, 2.0, 2.5, 3.0, 4.0, and 6.0 nM (*i.e.*, approximately 1.2- to 1.5-fold steps). Cells that grow well (*i.e.*, with little if any evidenced of cell death) in up to 2.5 nM HTI-286 were isolated about five months after initial exposure of the parental KB-3-1 cells line to that drug. These cells are designated KB-2.5-HTI. About ten months after the initial exposure of KB-3-1 cells to HTI-286, other cells were isolated that grew in up to 4.0 nM HTI-286. These cells are designated KB-4.0-HTI and are also

referred to here as KB-4-HTI cells. Both the KB-2.5-HTI and the KB-4.0-HTI cell lines are described in more detail below.

Additionally after stabilizing the KB-4.0-HTI line for 4 months, a subset of KB-4.0-HTI cells was removed from HTI-286 and maintained in normal (*i.e.*, drug free) growth media for 3 to 7 months prior to determining the resistance profile. This cell line is designated KB-4.0-HTI(-) and is also referred to here as KB-4-HTI(-) cells. The KB-4.0-HTI(-) cell line is described in more detail, *infra*. Finally, a parallel series of KB-3-1 cells was incubated with step-wise increasing concentrations of paclitaxel up to 15 nM. These cells, designated KB-15.0-PTX, are described elsewhere (Loganzo *et al.*, *Proc. Amer. Assoc. Cancer Res.* 2003, 44:1152 (abstract #5770)).

7.3. Resistance Profiles of HTI-286 Resistant Cell Lines

After selection for resistance to HTI-286, cell lines were characterized by assessing cell proliferation in the presence of various compounds to determine a resistance profile for each cell line, according to the procedures described above. Further characterization was performed by exposing HTI-286 resistant cells *in vivo* to HTI-286 and paclitaxel.

The drug resistance profile determined for each cell line evaluated in these experiments is set forth in **Table 1**, below.

Table 1: Drug Resistance Profiles of KB-3-1 Cells and HTI-286 Resistant KB Cells

	IC_{50} (nM) [1]		
Compound	**KB-3-1**	**KB-4.0-HTI**	**Relative resistance[2]**
Microtubule Depolymerizing Agents			
HTI-286	0.53 ± 0.05	6.50 ± 1.60	12.3
Hemiasterlin A	0.33 ± 0.05	4.38 ± 3.37	13.3
Dolastatin –10	0.03 ± 0.02	0.83 ± 0.33	27.7
Dolastatin –15	0.04 ± 0.05	0.49 ± 0.14	12.3
Maytansine	0.06 ± 0.00	0.84 ± 0.19	14.0
Rhizoxin	9.13 ± 2.17	98.37 ± 37.60	10.8
Vinblastine	0.73 ± 0.03	6.19 ± 1.26	8.5
Vinorelbine	1.22 ± 0.33	8.64 ± 2.94	7.1
Phomopsin A	671 ± 55	3529 ± 2988	5.3
Colchicine	6.64 ± 1.02	22.35 ± 0.07	3.4
Microtubule Polymerizing Agents			
Paclitaxel	2.66 ± 0.92	3.18 ± 0.62	1.2
Docetaxel	0.44 ± 0.20	1.25 ± 0.76	2.8
MAC-321	0.47 ± 0.14	2.54 ± 1.18	5.4
Epothilone B	0.63 ± 0.08	2.37 ± 2.20	3.8
Eleutherobin	17.41 ± 1.63	21.33 ± 4.40	1.2
DNA active drugs			
Topotecan	18.13 ± 2.98	1029 ± 541	56.8
Mitoxantrone	10.01 ± 6.50	430 ± 159	43.0
Camptothecin	14.86 ± 6.11	335 ± 157	22.5
Adriamycin	33.93 ± 21.19	552 ± 111	16.3
Bisantrene	119 ± 47	299 ± 91	2.5

1. Values shown are mean IC_{50} (nM) ± standard deviation.
2. Relative resistance is the ratio of the IC_{50} of the resistant cell line to the IC_{50} of the parental line.

Briefly, the KB-2.5-HTI cells are 9-fold resistant to HTI-286 (IC_{50} = 4.98 ± 1.18 nM) and are also highly cross-resistant to dolastatin-10 (26-fold) compared to the parental KB-3-1 cell line. Low resistance was observed to vinblastine and vinorelbine (5-fold). Low or minimal resistance was observed to colchicine (2.3-fold) and to the tubulin stabilizing agents paclitaxel (1.9-fold), docetaxel (3.2-fold), and epothilone B (3.2-fold).

However, these cells showed high resistance to several DNA damaging drugs, including camptothecin, topotecan, and mitoxantrone (18- to 44-fold).

Resistance profile data for the KB-4.0-HTI cells are also set forth in **Table 1** and are similar to data for the KB-2.5-HTI cells. However, resistance to HTI-286 is slightly higher in the KB-4.0-HTI cells compared to the KB-2.5-HTI cells. In particular, the KB-4.0-HTI cells are about 12- to 13- fold resistant to both HTI-286 and the highly related analog, hemiasterlin A.

The HTI-286 resistant cells also exhibit cross-resistance to several tubulin depolymerizing agents, including the Vinca alkaloids vinblastine and vinorelbine (7- to 9-fold), as well as drugs that competitively inhibit Vinca alkaloid binding to microtubules (maytansine and rhizoxin: 11- to 14-fold). Moderate to high levels of resistance were also observed for drugs that have been reported to bind to the Vinca-peptide binding domain in tubulin, including dolastatin-10 (28-fold), dolastatin-15 (12-fold), and phomopsin A (5-fold). Minimal resistance was observed to colchicine (3-fold), which binds to a site distinct from these other depolymerizing agents.

Low level resistance (1-5 fold) was observed for agents that induce microtubule polymerization, including three taxane analogs (paclitaxel, docetaxel, and MAC-321). Moderate to high level cross-resistance (16-57-fold) was observed to cytotoxic agents known to interact with DNA, with the exception of bisantrene where resistance increased only 3-fold.

To evaluate the stability of HTI-286 resistance, a subset of KB-4.0-HTI cells was removed from HTI-286 and maintained in normal (*i.e.*, drug free) growth media for 3 to 7 months prior to determining the drug resistance profile. The profile for these cells, which are designated KB-4.0-HTI(-), is also set forth in **Table 1**, above. Briefly, the KB-4.0-HTI(-) cells lost much of their resistance to HTI-286 and dolastatin-10, stabilizing at approximately 4-fold resistance to these drugs. In contrast, these cells retained their high level resistance to the DNA damaging drugs topotecan and mitoxantrone. Resistance to microtubule polymerizing agents was similar in both KB-4.0-HTI and KB-4.0-HTI(-) drugs. These results show that resistance to HTI-286 and dolastatin-10 is partially reversible after eliminating exposure to HTI-286

7.4. Evaluation of HTI-286 Resistance *In Vivo*

HTI-286 resistant cells were also evaluated *in vivo* to determine whether the cells' low level of drug resistance may be associated with a poor response to treatment with that

drug. Briefly, HTI-286 resistant cells were implanted subcutaneously in athymic mice. Cells from the parental KB-3-1 cell line were also implanted subcutaneously in identical mice as a control experiments. The transplanted tumors were allowed to attain a mass of between 80 and 120 mg, at which point the mice were treated with HTI-286, paclitaxel, or vehicle control.

Paclitaxel completely inhibits the growth of tumors derived from both the parental KB-3-1 and the KB-2.5-HTI cell lines (**Figures 1A** and **1B**, respectively). However, while HTI-286 inhibited growth of KB-3-1 tumors by 75% compared with saline-treated animals on day 28 (**Figure 1A**), little or no significant inhibitory effects were observed in tumors derived from the KB-2.5-HTI cells (**Figure 1B**). As explained above, cultures of the KB-2.5-HTI cells are also 9-fold resistant to HTI-286 *in vitro*, but are not highly resistant to paclitaxel. Hence, the results in this experiment demonstrate that the drug resistance properties observed *in vitro* for HTI-286 resistant cells are consistent with their drug resistance properties *in vivo*.

7.5. Resistance Mechanisms of HTI-286 Resistant Cells

Common resistance mechanisms for tubulin-binding drugs in resistant cells include the expression of drug efflux pumps such as the ABC transporters, MDR1 (ABCB1), MXR (ABCG2/BCRP), and MRP3 (ABCC3), and mutations in tubulin (Dumontet, *Expert Opin. Investig. Drugs* 2000, 9:779-788). This example describes experiments that examine such resistance mechanisms in HTI-286 resistant cells.

7.5.1 Expression of Drug Transport Proteins in HTI-286 Resistant Cells

Expression levels of the drug efflux pumps MDR1, MXR, and MRP3 were investigated in HTI-286 resistant cells using both real time RT-PCR and immunoblot analysis, as described in the Materials and Methods section, above.

MDR1 mRNA levels were not higher in either the KB-4.0-HTI or KB-4.0-HTI(-) cells compared to the KB-3-1 cell line from which these drug resistant cells were derived (**Figure 2A**). Indeed, MDR1 mRNA levels were actually lower in KB-4.0-HTI cells when measured with a highly sensitive real time RT-PCR method. Similarly, no MDR1 protein was detected by immunoblot analysis in either KB-3-1 or the HTI-286 resistant cells. These results differ substantially from the MDR1 mRNA and protein levels reported in KB-8-5 cells which are resistant to colchicine (Shen *et al.*, *J. Biol. Chem.* 1986, 261:7762-7770). Similarly, MDR1 mRNA and protein levels are highly elevated in

the paclitaxel resistant cell line KB-15.0-PTX. These data therefore indicate that theMDR1 efflux pump is not involved in resistance to HTI-286.

Because the HTI-286 resistant cells did exhibit substantial cross-resistant to compounds (*e.g.*, mitoxantrone and topotecan) that are known substrates of the MXR drug efflux pump, expression of that transporter was also investigated. MXR mRNA and protein were detected in all of the KB cells tested (including the non-resistant KB-3-1 cell line). However, these levels were substantially lower than those in another cell line (S1-M1-3.2) which is reported to be highly resistant to mitoxantrone due to overexpression of MXR (see Rabindran *et al., Cancer Res.* 1998, 58:5850-5858). MXR mRNA and protein levels remained unchanged in the KB-4.0-HTI and KB-4.0-HTI(-) cells compared to the parental KB-3-1 cell line (**Figure 2B**).

To verify these results, a functional assay was performed which evaluates the potential reversibility of MXR-mediated drug efflux. In particular, fumitrmorgin C (FTC) has been reported to resensitize MXR-expressing cells to the drugs mixtoxantrone and topotecan (Rabindran *et al., Cancer Res.* 1998, 58:5850-5858). When administered to KB-2.5-HTI cells, however, FTC failed to enhance sensitivity to HTI-286, mitoxantrone, or topotecan. However, FTC did enhance sensitivity of S1-M1-3.2 cells to mitoxantrone by 22-fold. Hence, these experiments indicate that the MXR efflux pump also is not involved in these cells' resistance to either HTI-286 or DNA damaging drugs.

Levels of MRP3 protein were found to be unchanged between the parental and the KB-4.0-HTI cells (**Figure 2C**). In contrast, high levels of MRP3 protein were detected in the positive control cell lines 2008/MRP3-8 (which is transfected with MRP3) and A549 (which has been previously shown to express the protein) (Kool *et al., Proc. Natl. Acad. Sci. USA* 1999, 96:6914-6919; Young *et al., Clin. Cancer Res.* 2001, 7:1798-1804).

In summary, the levels of the drug efflux pumps, MDR1, MXR, and MRP3, were not found to be elevated in HTI-286 resistant cell lines compared to the parental cell line. These data suggest that another efflux pump is removing drug from the cell interior.

7.5.2 HTI-286 Accumulation in Drug Resistant Cells

This example describes experiments that were performed to determine whether an alternative drug transporter (*i.e.*, a drug transporter other than MDR1, MXR, or MRP3) may contribute to HTI-286 resistance. In particular, these experiments measured the cellular accumulation of radiolabeled HTI-286 as described in the materials and methods

section, *supra*. Briefly, cells were incubated in the presence of either [^{3}H]-HTI-286 or [^{14}C]-paclitaxel for 2 hours and the level of drug remaining in those cells was determined by liquid scintillation counting of cell lysates.

The amount of radiolabeled HTI-286 remaining in KB-2.5-HTI and KB-4.0-HTI cells after the incubation period was 38% and 61% lower, respectively, than the amount in parental KB-3-1 cells, as indicated by the solid bars in **Figure 3**. In contrast, [^{3}H]-HTI-286 accumulation was unchanged in KB-4.0-HTI(-) cells compared with parental KB-3-1. No change in accumulation of [^{14}C]-paclitaxel was observed in HTI-286 resistant cell lines compared to the parental line (indicated by the unshaded bars in **Figure 3**), consistent with their lack of MDR1 expression. As a positive control, low accumulation of paclitaxel is observed in another cell line, known as KB-V1, that are reported to express high levels of the MDR1 efflux protein (Shen *et al.*, *J. Biol. Chem.* 1986, 261:7762-7770).

The low levels of HTI-286 accumulation in KB-4.0-HTI cells indicate that an alternative drug transporter may be present in those cells, and that this transporter may at least partly explain those cells' resistance to HTI-286.

7.5.3 Energy Dependence of HTI-286 Accumulation in Resistant Cells

This example describes experiments that further investigate the possible presence of a drug transporter in HTI-286 resistant cells. In particular, drug efflux pumps such as the ABC transporters are known to require ATP for drug efflux (Gottesman *et al.*, *Nature Reviews: Cancer* 2002, 2:48-58). Hence, these experiments investigate whether the low accumulation of HTI-286 in drug resistant cells is dependent upon ATP.

Briefly, sodium azide was administered to HTI-286 resistant cells as described in the Materials and Methods section, *supra*. The low accumulation of HTI-286 in KB-4.0-HTI cells was almost completely abolished in the presence of sodium azide, as shown by the unshaded columns in **Figure 4**. By contrast, sodium azide did not effect HTI-286 accumulation in either KB-3-1 or KB-4.0-HTI(-) cells, which are much less resistant to that drug. As a positive control, sodium azide was also administered to KB-V1 cells, and was found to partially reverse the low accumulation of HTI-286 in those cells (presumably by inhibition of the MDR1 protein which is known to be highly overexpressed in this cell line). Because sodium azide is understood to inhibit the production of ATP in cells (see for example, Schwoebel *et al.*, *J Cell Biol.* 2002, 157:963-

74), these experiments demonstrate that an ABC drug transporter, which is distinct from MDR1, MXR, and MRP3, prevents accumulation of HTI-286 in cells that are resistant to that drug.

7.5.4 Tubulin Mutations in HTI-286 Resistant Cells

This example describes experiments that isolate and sequence tubulin mRNA in drug resistant cells, to determine whether these cells contain mutations in tubulin that may at least partly account for their drug resistant phenotype. Specifically, total RNA was isolated from parent KB-3-1 cells and from drug resistant cells, and this RNA was reverse transcribed into cDNA. The cDNA was then PCR amplified and sequenced as described in the Materials and Methods section above.

An α-tubulin sequence corresponding to the sequence of human α1-tubulin (GenBank accession number BC017004) was observed. The human α1-tubulin sequence is shown in **Figure 5A**, along with the amino acid sequence it encodes **Figure 5B**.

No differences were detected in the predominant hM40 β-tubulin isotype isolated from KB-3-1, KB-4.0-HTI and KB-4.0-HTI(-) cells. However, a mutation in α-tubulin cDNA from both KB-4.0-HTI and KB-4.0-HTI(-) cells was detected. In particular, a single nucleotide change (GCT to TCT) was observed in codon 12 of α-tubulin cDNA isolated from these cells. This mutation produces a single amino acid change in the predicted α-tubulin protein sequence encoded by that cDNA. In particular, the mutations converts amino acid residue 12, which is an alanine in the wild-type human α-tubulin amino acid sequence (**Figure 5B**), to a serine.

Figures 6A and 6B illustrate a three-dimensional model of tubulin, obtained from the Protein Data Bank (Berman *et al.*, *Nucl. Acids Res.* 2000, 28:235-242) Accession No. 1JFF. The region containing the Ala12 to Ser mutation is expanded in **Figure 6B**. Inspection of this structure reveals that the mutation is proximal to the non-exchanging GTP (N) site at the interface between α- and β-tubulin (**Figure 6B**). This mutation is approximately 3 to 4 Å from the N-site GTP and is considered to be in direct contact with the nucleotide (Lowe *et al.*, *J. Mol. Bio.* 2001, 313:1045-1057). GTP binding to the N-site requires a divalent magnesium ion (Mg^{2+}) at this site (Correia *et al. J. Biol. Chem.* 1988, 263:10681-10686). Thus, this mutation could alter the binding of GTP and/or Mg^{2+} to tubulin. This mutation could also affect the longitudinal contacts between tubulin

subunits since the non-exchanging GTP (N) site is proximal to the interface between α- and β-tubulin.

cDNA sequences containing mixtures of the GCT and the altered TCT codons were consistently observed on chromatograms from these cells, even when different sequencing primers were used. Hence, the results indicate that each cell line expresses both a wild-type and mutated α-tubulin mRNA and/or that the cell lines contain a mixed population of cells (in which only some of the cells may contain the α-tubulin mutation). The α-tubulin mutation was not detected in the KB-2.5-HTI cells, indicating that this mutation is a later event in the selection for drug resistance.

8. REFERENCES CITED

Numerous references, including patents, patent applications and various publications, are cited and discussed in the description of this invention. The citation and/or discussion of such references is provided merely to clarify the description of the present invention and is not an admission that any such reference is "prior art" to the invention described here. All references cited and/or discussed in this specification (including references, *e.g.*, to biological sequences or structures in the GenBank, PDB or other public databases) are incorporated herein by reference in their entirety and to the same extent as if each reference was individually incorporated by reference.

WHAT IS CLAIMED IS:

1. An isolated cell derived from a parental cell that is a KB cell, wherein growth or proliferation of said isolated cell is resistant to a hemiasterlin compound, or to a derivative or analogue thereof.

2. An isolated cell line according to claim 1, wherein the parental cell is a KB-3-1 cell.

3. An isolated cell according to claim 1 wherein growth or proliferation of said isolated cell is resistant to HTI-286.

4. An isolated cell according to claim 1 wherein growth or proliferation of said cell is resistant to hemiasterlin A.

5. The isolated cell according to claim 3 wherein growth or proliferation of said isolated cell is at least 9-fold resistant to HTI-286 compared to the parental cell.

6. The isolated cell according to claim 3 wherein growth or proliferation of said isolated cell is at least 12-fold resistant to HTI-286 compared to the parental cell.

7. The isolated cell according to claim 3 wherein concentration of HTI-286 that inhibits growth or proliferation of said isolated cell by 50% (the IC_{50}) is at least about 5 nM.

8. The isolated cell according to claim 7 wherein the IC_{50} is at least about 6.5 nM.

9. The isolated cell according to claim 1, wherein growth or proliferation of said cells is additionally resistant to a compound selected from the group consisting of: vinblastine, vinorelbine, maytansine, rhizoxin, dolastatin-10, dolastatin-15, phomopsin A, docetaxel, comptothecin, topotecan, mitoxantrone, and bisantrene.

10. The isolated cell according to claim 1, in which there is a lower intracellular accumulation of the hemiasterlin compound, or the derivative or analogue thereof, in said isolated cell compared to in the parental cell.

11. The isolated cell according to claim 10, in which the intracellular accumulation of the hemiasterlin compound, or of the derivative or analogue thereof, in said isolated cell is at least 50% lower than in the parental cell.

12. The isolated cell according to claim 10 in which the intracellular accumulation of the hemiasterlin compound, or of the derivative or analogue thereof, in said isolated cell is at least 70% lower than in the parental cell.

13. An isolated cell according to claim 10 wherein said isolated cell exhibits an increased intracellular accumulation of the hemiasterlin compound, or of the derivative or analogue thereof, when incubated in the presence of an ATP inhibitor.

14. An isolated cell according to claim 13 wherein the ATP inhibitor is sodium azide.

15. An isolated cell according to claim 10 or 13 in which said cell does not express higher levels of a drug efflux pump than the parent cell, said drug efflux pump being selected from the group consisting of MDR1, MRP3, and MXR.

16. The isolated cell according to claim 1, which cell expresses a polypeptide comprising the amino acid sequence set forth in **Figure 5B** with an amino acid substitution at residue Ala12.

17. The isolated cell according to claim 16 wherein the amino acid substitutions is Ala12 → Ser.

18. The isolated cell according to claim 1, which cell expresses a nucleic acid that encodes a polypeptide having the amino acid sequence set forth in **Figure 5B** with an amino acid substitution at residue Ala12.

19. The isolated cell according to claim 18, wherein the amino acid substitution is Ala12 → Ser.

20. The isolated cell according to claim 1, which cell expresses a nucleic acid comprising the nucleotide sequence set forth in **Figure 5A** with at least one substitution at any one or more of nucleotides 34-36.

21. The isolated cell according to claim 20, wherein the at least one substitution at nucleotides 34-36 is GCT → TCT.

22. An isolated cell derived from a parental cell that is a KB cell and having a lower intracellular accumulation of the hemiasterlin compound, or the derivative or analogue thereof, compared to in the parental cell.

23. The isolated cell according to claim 16, in which the intracellular accumulation of the hemiasterlin compound, or of the derivative or analogue thereof, in said isolated cell is at least 50% lower than in the parental cell.

24. The isolated cell according to claim 16 in which the intracellular accumulation of the hemiasterlin compound, or of the derivative or analogue thereof, in said isolated cell is at least 70% lower than in the parental cell.

25. An isolated cell according to claim 16 wherein there is an increased intracellular accumulation of the hemiasterlin compound, or of the derivative or analogue thereof, in said isolated cell when incubated in the presence of an ATP inhibitor.

26. An isolated cell according to claim 19 wherein the ATP inhibitor is sodium azide.

27. An isolated cell according to claim 16 or 19 in which said cell does not express higher levels of a drug efflux pump than the parent cell, said drug efflux pump being selected from the group consisting of MDR1, MRP3, MXR.

28. An isolated cell which expresses a polypeptide comprising the amino acid sequence set forth in **Figure 5B** with an amino acid substitution at residues Ala12.

29. The isolated cell according to claim 28 wherein the amino acid substitutions in Ala12 → Ser.

30. An isolated cell which expresses a nucleic acid that encodes a polypeptide having the amino acid sequence set forth in **Figure 5B** with an amino acid substitution at residue Ala12.

31. The isolated cell according to claim 30, wherein the amino acid substitution is Ala12 → Ser.

32. An isolated cell which expresses a nucleic acid comprising the nucleotide sequence set forth in **Figure 5A** with at least one substitution at any one or more of nucleotides 34-36.

33. The isolated cell according to claim 32, wherein the at least one substitution at nucleotides 34-36 is GCT → TCT.

34. An isolated cell KB-2.5-HTI.

35. An isolated cell KB-4.0-HTI.

36. An isolated cell KB-4.0-HTI(-).

Figure 1A

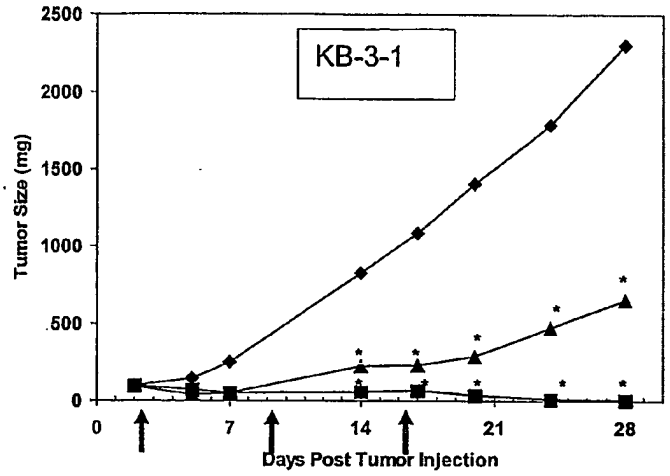


Figure 1B

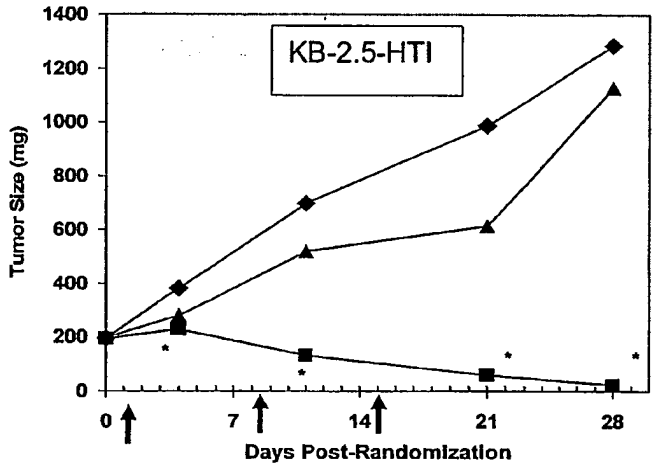


Figure 2A

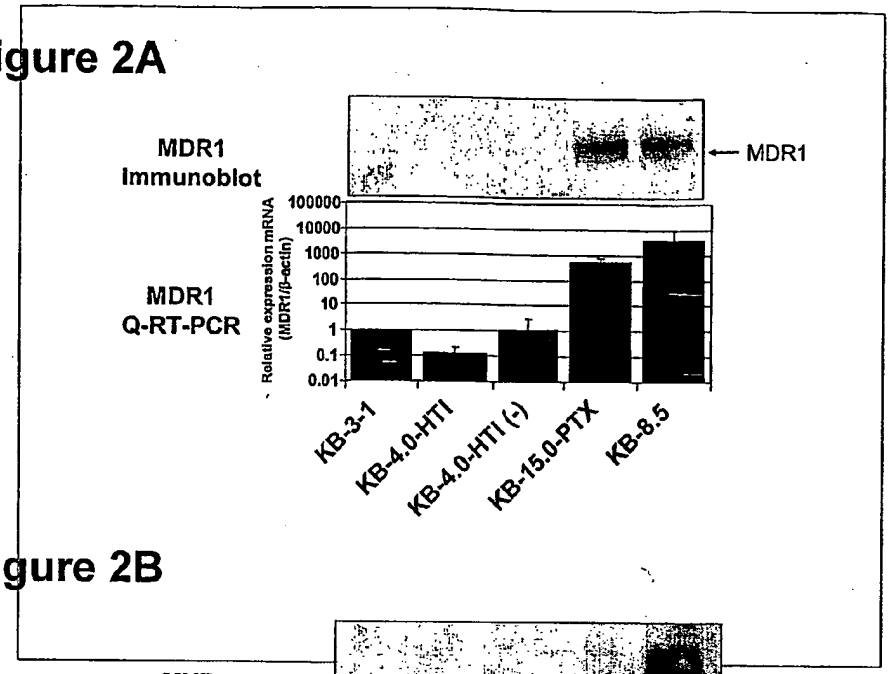


Figure 2B

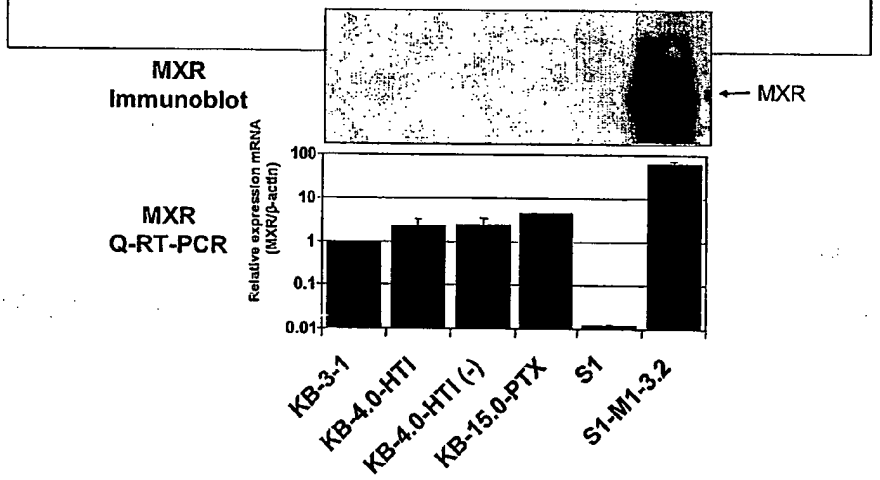
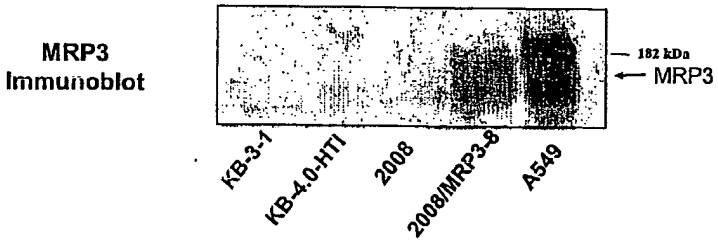
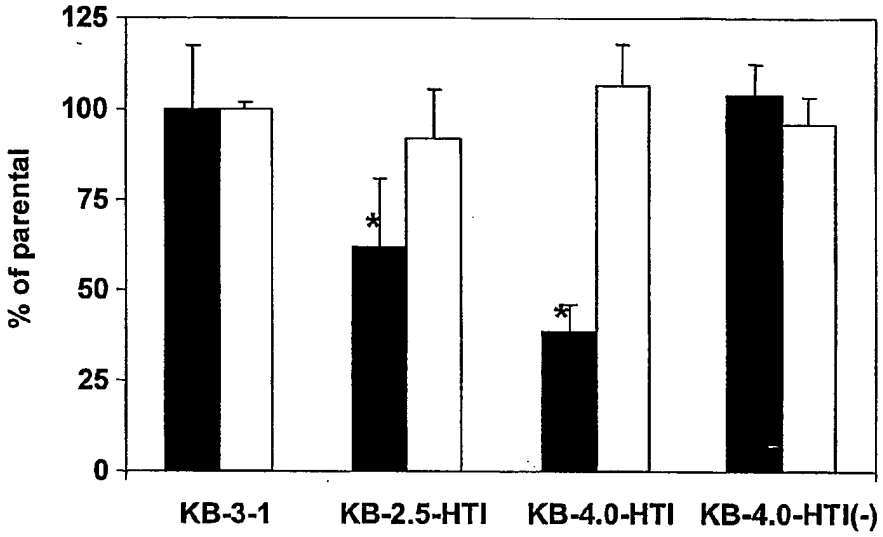


Figure 2C





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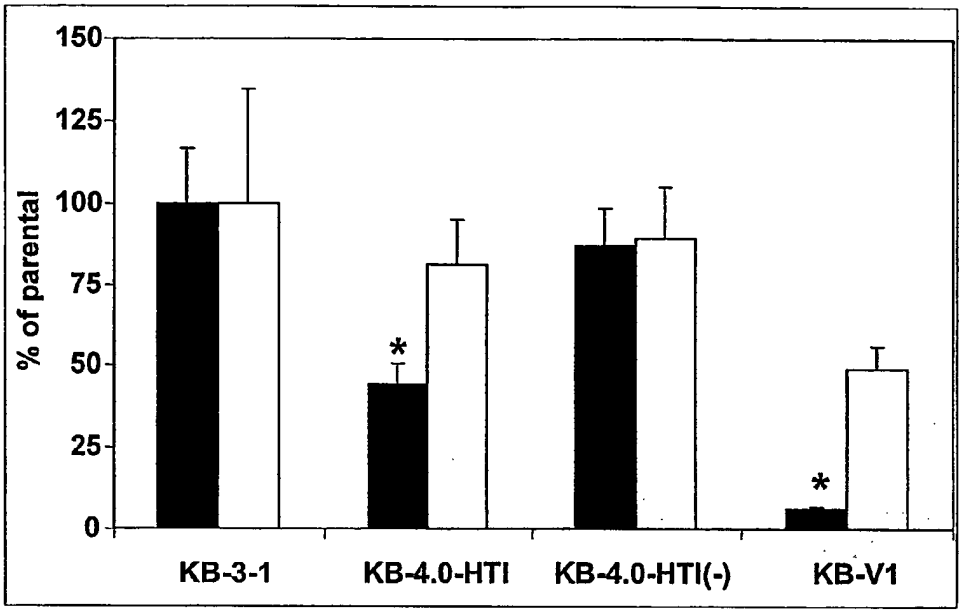

Figure 4

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1    atgcgtgagt gcatctccat ccacgttggc caggctggtg tccagattgg caatgcctgc
61   tgggagctct actgcctgga acacggcatc cagcccgatg gccagatgcc aagtgacaag
121  accattgggg gaggagatga ctccttcaac accttcttca gtgagacggg cgctggcaag
181  cacgtgcccc gggctgtgtt tgtagacttg gaacccacag tcattgatga agttcgcact
241  ggcacctacc gccagctctt ccaccctgag cagctcatca caggcaagga agatgctgcc
301  aataactatg cccgagggca ctacaccatt ggcaaggaga tcattgacct tgtgttggac
361  cgaattcgca agctggctga ccagtgcacc ggtcttcagg gcttcttggt tttccacagc
421  tttggtgggg gaactggttc tgggttcacc tccctgctca tggaacgtct ctcagttgat
481  tatggcaaga aatccaagct ggagttctcc atttacccgg caccccaggt ttccacagct
541  gtagttgagc cctacaactc catcctcacc acccacacca ccctggagca ctctgattgt
601  gccttcatgg tagacaatga ggccatctat gacatctgtc gtagaaacct cgatatcgag
661  cgcccaacct acactaacct taaccgcctt attagccaga ttgtgtcctc catcactgct
721  tccctgagat ttgatggagc cctgaatgtt gacctgacag aattccagac caacctggtg
781  ccctaccccc gcatccactt ccctctggcc acatatgccc ctgtcatctc tgctgagaaa
841  gcctaccatg aacagctttc tgtagcagag atcaccaatg cttgctttga gccagccaac
901  cagatggtga aatgtgaccc tcgccatggt aaatacatgg cttgctgcct gttgtaccgt
961  ggtgacgtgg ttcccaaaga tgtcaatgct gccattgcca ccatcaaaac caagcgcagc
1021 atccagtttg tggattggtg ccccactggc ttcaaggttg gcatcaacta ccagcctccc
1081 actgtggtgc ctggtggaga cctggccaag gtacagagag ctgtgtgcat gctgagcaac
1141 accacagcca ttgctgaggc ctgggctcgc ctggaccaca agtttgacct gatgtatgcc
1201 aagcgtgcct ttgttcactg gtacgtgggt gaggggatgg aggaaggcga gttttcagag
1261 gcccgtgaag atatggctgc ccttgagaag gattatgagg aggttggtgt ggattctgtt
1321 gaaggagagg gtgaggaaga aggagaggaa tactaa
```

Figure 5A

```
1   MRECISIHVG QAGVQIGNAC WELYCLEHGI QPDGQMPSDK TIGGGDDSFN TFFSETGAGK
61  HVPRAVFVDL EPTVIDEVRT GTYRQLFHPE QLITGKEDAA NNYARGHYTI GKEIIDLVLD
121 RIRKLADQCT GLQGFLVFHS FGGGTGSGFT SLLMERLSVD YGKKSKLEFS IYPAPQVSTA
181 VVEPYNSILT THTTLEHSDC AFMVDNEAIY DICRRNLDIE RPTYTNLNRL ISQIVSSITA
241 SLRFDGALNV DLTEFQTNLV PYPRIHFPLA TYAPVISAEK AYHEQLSVAE ITNACFEPAN
301 QMVKCDPRHG KYMACCLLYR GDVVPKDVNA AIATIKTKRS IQFVDWCPTG FKVGINYQPP
361 TVVPGGDLAK VQRAVCMLSN TTAIAEAWAR LDHKFDLMYA KRAFVHWYVG EGMEEGEFSE
421 AREDMAALEK DYEEVGVDSV EGEGEEEGEE Y
```

Figure 5B

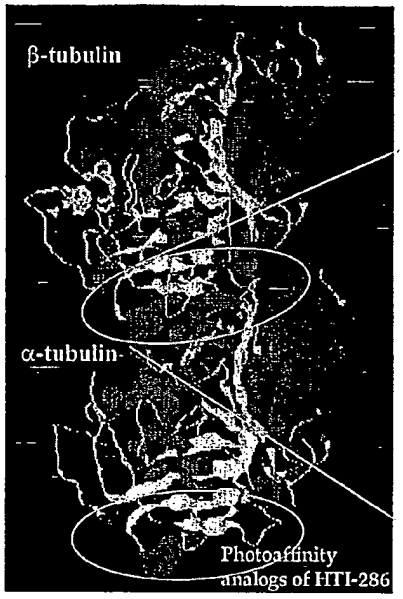

Figure 6A

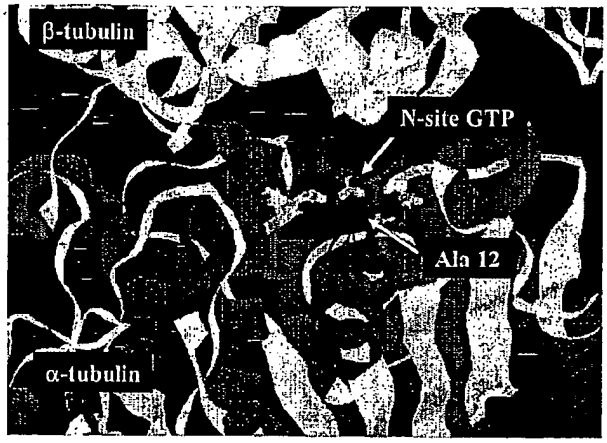

Figure 6B

```
Htub1/1-451          MRECISVHVGQA_GVQIGNACWELFCLEHGIQADGTFDAQASKINDDDSFTTFFSETGNGK
Htub2/1-451          MRECISIHVGQA_GVQIGNACWELYCLEHGIQPDGQMPSDKTIGGGDDSFNTFFSETGAGK
ptub1/1-451          -RECISVHVGQA_GVQMGNACWELYCLEHGIQPDGQMPSDKTIGGGDDSFTTFFCETGAGK
ptub2/1-451          ---------GQA_GVQIGNACWELYCLEHGIQPDGQMPSDKTIGGGDDSFNTFFSETGAGK
motub1/1-451         MRECISVHVGQA_GVQIGNACWELFCLEHGIQADGTFGTQASKINDDDSFTTFFSETGNGK
hamtub1/1-451        MRECISIHVGQA_GVQIGNACWELYCLEHGIQPDGQMPSDKTIGGGDDSFNTFFSETGAGK
rattub1/1-451        MRECISIHVGQA_GVQIGNACWELYCLEHGIQPDGQMPSDKTIGGGDDSFNTFFSETGAGK
chicktub1/1-451      MRECISVHIGQA_GVQIGNACWELFCLEHSIQPDGTFSDPPSS---DDSFATFFRETSMSK
frogtub1/1-451       MRECISIHVGQA_GVQIGNACWELYCLEHGIQPDGQMPSDKTIGGGDDSFNTFFSETGAGK

Htub1/1-451          HVPRAVMIDLEPTVVDEVRAGTYRQLFHPEQLITGKEDAANNYARGHYTVGKESIDLVLD
Htub2/1-451          HVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLITGKEDAANNYARGHYTIGKEIIDLVLD
ptub1/1-451          HVPRAVFVDLEPTVIDEIRNGPYRQLFHPEQLITGKEDAANNYARGHYTIGKEIIDPVLD
ptub2/1-451          HVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLITGKEDAANNYARGHYTIGKEIIDLVLD
motub1/1-451         HVPRAVMVDLEPTVVDEVRAGTYRQLFHPEQLITGKEDAANNYARGHYTVGKESIDLVLD
hamtub1/1-451        HVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLITGKEDAANNYARGHYTIGKEIIDLVLD
rattub1/1-451        HVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLITGKEDAANNYARGHYTIGKEIIDLVLD
chicktub1/1-451      YVPRAIMVDLEPTVVDEVRTGTYRHLFHPEQLITGKEDAANNYARGHYTVGKDKVDMVSD
frogtub1/1-451       HVPRAVFVDLEPTVIDEVRTGTYRQLFHPEQLITGKEDAANNYARGHYTIGKEIIDLVLD

Htub1/1-451          RIRKLTDACSGLQGFLIFHSFGGGTGSGFTSLLMERLSLDYGKKSKLEFAIYPAPQVSTA
Htub2/1-451          RIRKLADQCTRLQGFLVFHSFGGGTGSGFTSLLMERLSVDYGKKSKLEFSIYPAPQVSTA
ptub1/1-451          RIRKLSDQCTGLQGFLVFHSFGGGTGSGFTSLLMERLSVDYGKKSKLEFSIYPAPQVSTA
ptub2/1-451          RIRKLADQCTGLQGFLVFHSFGGGTGSGFTSLLMERLSVDYGKKSKLEFSIYPAPQVSTA
motub1/1-451         RIRKLTDACSGLQGFLIFHSFGGGTGSGFTSLLMERLSLDYGKKSKLEFAIYPAPQVSTA
hamtub1/1-451        RIRKLADQCTGLQGFLVFHSFGGGTGSGFTSLLMERLSVDYGKKSKLEFSIYPAPQVSTA
rattub1/1-451        RIRKLADQCTGLQGFLVFHSFGGGTGSGFTSLLMERLSVDYGKKSKLEFSIYPAPQVSTA
chicktub1/1-451      RIRKLADSCSGLQGFLIFHSFGGGTGSGFTSLLMERLSVEYGKKSKLEFAIYPAPQASSA
frogtub1/1-451       RIRKLADQCTGLQGFLVFHSFGGGTGSGFTSLLLERLSVDYGKKSKLEFAIYPAPQVSTA

Htub1/1-451          VVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLISQIVSSITA
Htub2/1-451          VVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLISQIVSSITA
ptub1/1-451          VVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLISQIVSSITA
ptub2/1-451          VVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQIVSSITA
motub1/1-451         VVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLISQIVSSITA
hamtub1/1-451        VVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLISQIVSSITA
rattub1/1-451        VVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLIGQIVSSITA
chicktub1/1-451      VVEPYNSVLTTHTTLEHSDCVFMVDNEAIYDICHRNLDIERPTYTNLNRLISQIVSSITA
frogtub1/1-451       VVEPYNSILTTHTTLEHSDCAFMVDNEAIYDICRRNLDIERPTYTNLNRLISQIVSSITA
```

Figure 7

```
Htub1/1-451        SLRFDGALNVDLTEFQTNLVPYPRIHFPLVTYAPIISAEKAYHEQLSVAEITSSCFEPNS
Htub2/1-451        SLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVADITNACFEPAN
ptub1/1-451        SLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVAEITNACFEPAN
ptub2/1-451        SLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVAEITNACFEPAN
motub1/1-451       SLRFDGALNVDLTEFQTNLVPYPRIHFPLVTYAPIISAEKAYHEQLSVAEITSSCFEPNS
hamtub1/1-451      SLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLTVAEITNACFEPAN
rattub1/1-451      SLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLSVAEITNACFEPAN
chicktub1/1-451    SLRFDGALNVDLTEFQTNLVPFPRIHFPLVTYAPIISSDRAYHEQLSVAEITSSCFEPNN
frogtub1/1-451     SLRFDGALNVDLTEFQTNLVPYPRIHFPLATYAPVISAEKAYHEQLTVADITNACFEPAN

Htub1/1-451        QMVKCDPRHGKYMACCMLYRGDVVPKDVNVAIAAIKTKRTIQFVDWCPTGFKVGINYQPP
Htub2/1-451        QMVKCDPGHGKYMACCLLYRGDVVPKDVNAAIATIKTKRTIQFVDWCPTGFKVGINYQPP
ptub1/1-451        QMVKCDPRHGKYMACCLLYRGDVVPKDVNAAIAAIKTKRSIQFVDWCPTGFKVGINYQPP
ptub2/1-451        QMVKCDPRHGKYMACCLLYRGDVVPKDVNAAIATIKTKRTIQFVDWCPTGFKVGINYQPP
motub1/1-451       QMVKCDPRHGKYMACCMLYRGDVVPKDVNVAIAAIKTKRTIQFVDWCPTGFKVGINYQPP
hamtub1/1-451      QMVKCDPRHGKYMACCLLYRGDVVPKDVNAAIATIKTKRTIQFVDWCPTGFKVGINYQPP
rattub1/1-451      QMVKCDPRHGKYMACCLLYRGDVVPKDVNAAIATIKTKRTIQFVDWCPTGFKVGINYQPP
chicktub1/1-451    QMVKCDPQQGKYMACCMLYRGDVVPKDVNVAIAAIKTNRSLQFVDWCPTGFKVGINYQPP
frogtub1/1-451     QMVKCDPRHGKYMACCLLYRGDVVPKDVNAAIATIKTKRSIQFVDWCPTGFKVGINYQPP

Htub1/1-451        TVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGMEEGEFSE
Htub2/1-451        TVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGMEEGEFSE
ptub1/1-451        TVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGMEEGEFSE
ptub2/1-451        TVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGMEEGEFSE
motub1/1-451       TVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGMEEGEFSE
hamtub1/1-451      TVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGMEEGEFSE
rattub1/1-451      TVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGMEEGEFSE
chicktub1/1-451    IPTPGGDLAQVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVSEGMEEGEFAE
frogtub1/1-451     TVVPGGDLAKVQRAVCMLSNTTAIAEAWARLDHKFDLMYAKRAFVHWYVGEGMEEGEFSE

Htub1/1-451        AREDLAALEKDYEEVGTDSFEEE--NEGEEF
Htub2/1-451        AREDMAALEKDYEEVGVDSVEGEGEEEGEEY
ptub1/1-451        AREDMAALEKDYEEVGIDSYEDE--DEGEE-
ptub2/1-451        AREDMAALEKDYEEVGVDSVEGEGEEEGEEY
motub1/1-451       AREDLAALEKDYEEVGTDSFEEE--NEGEEF
hamtub1/1-451      AREDMAALEKDYEEVGADSAEGD--DEGEEY
rattub1/1-451      AREDMAALEKDYEEVGVDSVEGEGEEEGEEY
chicktub1/1-451    AREDLAALEKDYDEVATDLFEDE-NEAGDS-
frogtub1/1-451     AREDMAALEKDYEEVGADSADAE--DEGEEY
```

Figure 7 (continued)

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(71) Applicant *(for all designated States except US)*: WYETH [US/US]; Five Giralda Farms, Madison, NJ 07940 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: GREENBERGER, Lee, M. [US/US]; 253 Midland Avenue, Montclair, New Jersey 07042 (US).

(74) Agent: FEHLNER, Paul, F.; Darby & Darby P.C., P.O. Box 5257, NY 10150-5257 (US).

(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

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(54) Title: CELLS RESISTANT TO CHEMOTHERAPEUTIC COMPOUNDS AND USES THEREOF

(57) Abstract: The present invention provides cell lines that are resistant to anti-tubulin drugs and, in particular, are resistant to hemiasterlins and/or the hemiasterlin derivative HTI 286. Methods are also provided which use these cell lines to screen for and identify drugs (particularly anti-tubulin drugs) that effectively inhibit the growth and/or replication of cells that are resistant to hemiasterlins and other anti-tubulin drugs such as HTI-286. The invention additionally provides methods that use these cell lines to characterize antitubulin drugs.

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INTERNATIONAL SEARCH REPORT

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PCT/US03/37392

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12N 5/08
US CL : 435/366
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/366

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
conference abstract

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PORUCHYNSKY et al. Tumor Cells Resistant to a Tubulin-Depolymerizing Hemiasterlin Analog, WAY-174286, Display Different Resistance Mechanisms, Including Mutations in alpha-or beta-Tubulin. November 2001, Clincal cancer research, Vol. 7, No. 11, supp. Pages: 3810S-3810S. (abstract)	1-36

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 October 2004 (25.10.2004)	03 NOV 2004
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US Commissioner of Patents P.O. Box 1450 Alexandria, Virginia 22313-1450 Facsimile No. (703) 305-3230	Authorized officer Jeffrey Siew Telephone No. 571-272-1600

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INTERNATIONAL SEARCH REPORT

PCT/US03/37392

Continuation of B. FIELDS SEARCHED Item 3:
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Public database: medline

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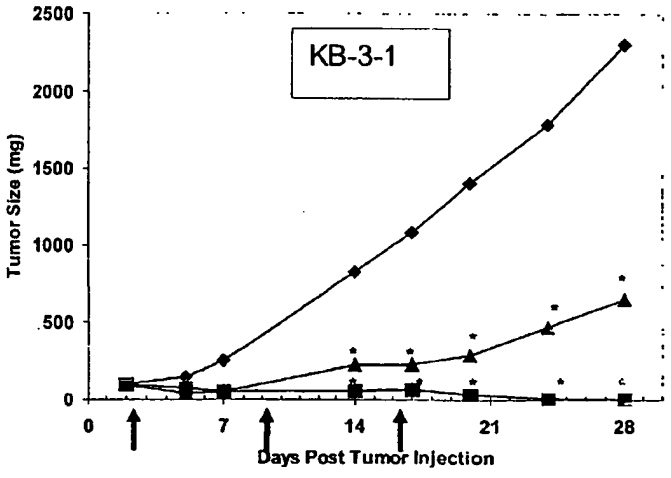

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